Filament structure and phosphatase activity in the Rivulariaceae

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Filament Structure and Phosphatase Activity in the Rivulariaceae

by

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

ABSTRACT

A study was carried out on phosphatase activity, phosphate uptake and its relationship to hair formation in the Rivulariaceae. The Rivulariaceae was chosen as it is a widespread taxon, where hair formation is a common occurrence, and previous studies indicated that they originate from environments where a large proportion of the phosphorus (P) is present as organic P. It seems possible that hair-forming Rivulariaceae are especially well adapted to utilize organic P.

Initially 51 axenic cyanobacterial strains, from 10 genera, were screened for yields using organic P sources and for cell-bound and extracellular phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities. All strains exhibited detectable inducible PMEase activities, and highest cell-bound PMEase activities were in hair-forming Rivulariaceae. Synechococcus had significantly low cell-bound phosphatase activities and five strains were unable to hydrolyze phytic acid. PDEase activities were lower compared to PMEase activities in all strains. Strains isolated from deepwater rice habitats had significantly higher levels of PDEase activity.

In the three Calothrix strains tested, Calothrix 202, 550 and 603, inducible phosphatase activities were similar whether the P source was inorganic or organic. PMEase synthesis in these strains began when cellular P (% dry wt) values were in the range 0.60 - 1.0%.

Differences in the influence of environmental variables on cell-bound and extracellular PMEase activities in hair-forming Calothrix 550 were slight, suggesting that PMEases in the two fractions had a common origin. Of the eleven ions tested Ca had the most pronounced stimulatory effect on PMEase activity. Localization of enzyme activity in Calothrix 550 suggested that the enzyme was bound to a surface. Partial purification of an extracellular PMEase fraction detected four bands of PMEase activity on a non-denaturing polyacrylamide gel. Three of the four bands were associated with carbohydrate and the bands were not extractable by mechanical means.

Localization of PMEase activity in hair-forming strains by azo dye (naphthol AS-MX) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) showed that PMEase activity was associated with hair cells. Phosphate uptake experiments with Calothrix 253 and 550 suggested that uptake at high external phosphate concentrations was located in hair cells.

NaCl, above 67.5 mM, inhibited hair formation and subsequently phosphatase activity in Calothrix 253 and 690. Addition of mannitol or sorbitol had no effect on hair formation, suggesting inhibition of hair formation was not an osmotic effect. Removal of P-deficient cultures from saline to freshwater media led to a marked synchronization of hair formation (in 90% of trichomes) and increase in cell-bound PMEase activity. Localization of cell-bound PMEase activity by light microscopy, using naphthol AS-MX, detected activity in the hair cells.

ABBREVIATIONS

°C	degrees Celsius
g	gramme
mg	milligramme
µg	microgramme
dry wt	dry weight
l	litre
ml	millilitre
µl	microlitre
m	metre
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre
d	day
h	hour
min	minute
s	second
M	molar
mM	millimolar
µm	micromolar
µmol	micromole
P	phosphorus
P_i	inorganic phosphate
FRP	filtrable reactive phosphorus
TFP	total filtrable phosphorus
PMEase	phosphomonoesterase
PDEase	phosphodiesterase
APA	alkaline phosphatase activity
ρNPP	ρ-nitrophenyl phosphate
bis-ρNPP	bis (ρ-nitrophenyl) phosphate
MNP	bispyridinium 2-methoxy-4-(2'nitrovinyl)-phenyl phosphate
K_m	Michaelis-Menten constant
V_{max}	maximum rate
AMeP	2-amino-2-methyl-1-propanol
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
DMG	3,3-dimethylglutaric acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
PAR	photosynthetically active radiation
n	number of samples
N.S	not significant
P	probability
S.D.	standard deviation

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The Rivulariaceae are one of the most morphologically diverse families in the cyanobacteria. Many filamentous forms in the Rivulariaceae have the ability to form hair cells at a particular stage in their life cycle.

Sinclair (1977) and Livingstone et al. (1983) suggested that hairs and possible associated phosphatase activities resulted from phosphate deficiency, although no one role has been attributed to hairs in the Rivulariaceae or any other cyanobacteria.

1.2 RIVULARIACEAE

The most recent description of the Rivulariaceae is in Bergey's Manual of Systematic Bacteriology volume III (Staley et al., 1989), which uses Calothrix parietina (D550) as the reference strain. Desikachary (1959) defined the Rivulariaceae as, "trichomes with a single row of cells, apices generally attenuated or tapering in a hair, unbranched or false branched, sometimes with a distinct intercalary meristematic zone and trichothallic growth; hair with elongated more or less vacuolated cells; heterocysts present or absent, when present basal, intercalary heterocysts also present in some; hormogonia present; akinetes present or absent, when present single or in series." Geitler (1932) used a similar definition and included 12 genera in his description. This is summarized in Table 1.1 (Whitton 1987).

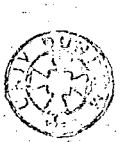


Table 1.1 The principal features of genera in the Rivulariaceae; status of genera in brackets doubtful (Whitton, 1987)

	Heterocyst	Mature trichome tapered at both ends	Cellular hair	Akinete	Filaments in a common gelatinous mucilage	Hemispherical or spherical colony
Amphithrix	-	-	? +	-	-	-
Calothrix	+ + +	-	+ +	+	+	+
Dichothrix	+ + +	-	+ + +	-	-	+
Gardnerula	+ + +	-	+ + +	-	-	-
Gloeotrichia	+ + +	-	+ + +	+ + +	+ + +	+ + +
Hammatoidea	-	+ + +	? -	-	-	-
Homoeothrix	-	-	+ +	-	-	+ +
Isactis	+ + +	-	+ + +	-	+ + +	
(Leptochaete)	-	-	+	-	-	-
Rivularia	+ + +	-	+ + +		+ + +	+ +
Sacconema	+ + +	-	+ + +	+	+ + +	-
(Tapinothrix)	-	-	-	-	-	-

+ + +, feature of genus: requires observation for identification.
+ +, widespread feature in genus, but sometimes absent; absence in at least some cases probably genetic.
+, occasional feature in genus.
-, feature absent in genus.

1.21 Morphological features of the Rivulariaceae

All species are thought to be able to form hormogonia. The hormogonia develop into tapered trichomes, which are aggregated in some way and in Gloeotrichia and Rivularia well ordered spherical and hemispherical colonies are formed. A hair may eventually develop at the end of the tapered trichomes. Population growth in the form of new trichomes mainly takes place through the release of hormogonia from the apical part of a mature trichome. However in forms with spherical or hemispherical colonies false branching can lead to the formation of new tapered trichomes.

The presence of a hair at the end of a trichome is the nearest to a distinctive feature within the family. However, hairs are also present in some or all species of Mastigocoleus, Nostochopsis, Brachytrichia and Kyrtuthrix (Whitton, 1987).

1.211 Hormogonia

The hormogonia are parallel lengths of cells, which exhibit gliding motility and develop in the meristematic zone at the apex of the trichome (Geitler, 1932). Hormogonia are released by the production of biconcave separation discs (Fritsch, 1945). During hormogonial release, the hairs, if present, are shed. After release, few, if any, cell divisions occur before further differentiation of the hormogonia into a mature trichome takes place. Gas vacuoles are present in young hormogonia of Calothrix and Gloeotrichia, although they are lost during differentiation.

1.212 Trichome growth

Growth in Rivulariaceae is typically meristematic, i.e. cell division is confined to a particular part of the trichome. In forms with heterocysts and hairs division occurs between the two, often just below the hair (trichothallic growth - Fritsch, 1945). The cells of the meristematic region

in some forms are wider than the basal cells giving the appearance of a spindle shaped swelling below the hair (Whitton, 1987).

Morphological polarity was largely or entirely lost when heterocystous Rivulariaceae were transferred to a medium with combined nitrogen. 33 out of 34 strains studied by Sinclair and Whitton (1977b) stopped heterocyst formation in the presence of combined nitrogen. 19 strains lost their taper and in the remaining strains there was a mixture of tapered and non-tapered trichomes. Hair frequency and length were reduced markedly in three strains.

1.213 Heterocyst

The presence of a heterocyst is a diagnostic feature at the generic level (Table 1.1), although a high concentration of combined nitrogen can suppress its formation (Sinclair and Whitton, 1977b). Where heterocysts are present, they are mostly terminal, i.e. with a polar nodule on one side. The terminal heterocyst developing at the end of a hormogonium in several strains of Calothrix parietina differentiate from the cell which is nearest the parent trichome before the hormogonium is released (Whitton, 1987). This polarity of the trichome is therefore established before it is observable with the light microscope.

Intercalary heterocysts develop occasionally in some Calothrix species (Geitler, 1932). In C. brevissima (Rai et al., 1978) bipolar heterocysts developed in trichomes which had grown in the presence of ammonia and then deprived of it. Subsequently one polar nodule flattened and the adjoining cell died, forming a separation disc which caused fragmentation of the filament. In comparison with other heterocystous families, heterocysts tend to form a smaller proportion of the total cells in the Rivulariaceae.

A widespread feature in the Rivulariaceae is the presence of green and blue-green heterocysts. This is particularly common in Calothrix and Gloeotrichia isolates from rice fields (Whitton et al., 1987). Other

differences also occur among heterocysts in the Rivulariaceae. In a Bangladesh Calothrix (D603) strain carboxysomes occurred in mature heterocysts (Whitton et al., 1987). There are a number of records of heterocyst germination in the Rivulariaceae (Desikachary, 1946), although this feature has been noted in other families.

1.214 Hair

Bornet and Flahault (1886a,b) defined the cyanobacterial hair as "a series of narrow, elongated cells, containing very little protoplasm, and incapable of further growth". As this definition confuses different features, Sinclair and Whitton (1977a) redefined the hair as a region of the trichome where the cells are narrow, elongated, highly vacuolated and usually apparently colourless. Geitler (1932) described the presence of hairs in the genera Calothrix, Dichothrix, Gloeotrichia, Isactis, Polythrix, Rivularia, Sacconema, Ammatoidea, Homoeothrix, and Leptochaete of the Rivulariaceae. Hairs are also present in a number of genera not in the Rivulariaceae, Loefgrenia (Loefgreniaceae), Mastigocoleus and Nostochopsis (Nostochopsidaceae) and Brachytrichia and Kyrtuthrix (Mastigocladaceae).

Of 36 strains of Rivulariaceae in culture (Sinclair and Whitton, 1977a) 13 formed hairs. In all cases abundant hair increase occurred under conditions of phosphate deficiency. Fe deficiency led to hair formation in 8 strains and Mg deficiency in one strain. Of 78 strains with quite detailed descriptions in the taxonomic literature, hairs are present in 52 strains, absent in 16 and data inadequate for comment in 10 (Kirkby and Whitton, 1976). The question arises as to what extent the lack of hairs is merely an environmental effect. Hairs were formed in Calothrix parietina when the average value of cellular phosphorus (P) fell below 1% dry weight, which is a concentration found in healthy filaments (Livingstone et al., 1983).

The effect of P on hair formation and Rivulariaceae morphology is quite dramatic and it has been suggested that the P status of the environment may play an important role in the growth of Rivulariaceae (Whitton, 1987). The changes in morphology related to the P status of C. parietina have been described by Livingstone and Whitton (1984). Four distinct morphological stages were recognised. Stage I commenced with the release of a hormogonium and ended with the formation of a heterocyst. Stage II commenced with formation of a mature heterocyst, followed by tapering of the trichome. Under P rich conditions this tapered trichome can continue to give rise to hormogonia, but with increasing P deficiency the terminal cells begin to differentiate into a hair (Stage III). When phosphate is added to trichomes with hairs (Stage IV) a further characteristic stage of events takes place. Hormogonia develop at the apical end of the chlorophyll containing part of the trichome; the hair falls off and hormogonia are released by gliding from the sheaths.

Cytological and ultrastructural studies of the hair mainly describe a progressive loss of cell constituents from "vegetative" to typical hair cell. Geitler (1932) suggested that a gradual transition was common, however the transition can be very abrupt with elongation and vacuolation occurring over a short distance (Sinclair, 1977). Ueda (1971b) showed that the hair cell adjacent to the vegetative cells had between two to five times less DNA. Miller and Lang (1971) showed that the hair cell was mainly intrathylakoidal space, with the remaining part of the thylakoid system existing as single membranes bounding the remaining interthylakoidal cytoplasm in Gloeotrichia sp. There was an increase in cyanophycin granulation, and a decrease in polyglucoside granulation as the cells aged; in the oldest cells all inclusions except lipid globules and carboxysomes were absent.

Douglas (1979) and Wood (1984) showed that all cytoplasmic inclusions other than membranes were absent in a range of Calothrix and Rivularia strains.

However it was shown by Smith and Peat (1967) that gas vacuoles persist in hairs of Gloeotrichia echinulata.

Although cell vacuolation is the dominant feature in the hairs of Rivulariaceae, it can occur in other cyanobacteria. The formation of vacuoles is a common response in older cells in response to extreme conditions (Desikachary, 1959). The apical cells of subaerial species are sometimes vacuolated, perhaps as a result of desiccation, since vacuoles are not present in submerged forms. In Oscillatoria borneti and O. pseudogeminata (Ueda, 1971a) small vacuoles developed giving the cytoplasm an alveolate appearance. These keritomic vacuoles (Keritomie: Geitler, 1960) were caused by swelling of the intrathylakoidal space, and their development was accelerated by increasing the light intensity. Keritomie occurs as a pathological symptom in otherwise normal cells, still capable of division, and it is reversible. However vacuolation in Rivulariaceae is a normal stage in their life cycle, and is irreversible after a certain stage. A reduction in granulation and colour has also been noted in lateral branches of members of the Stigonemataceae (Thurston and Ingram, 1971).

From the above examples it shows that hair formation is not unique to the Rivulariaceae. However the Rivulariaceae are the family of cyanobacteria which show the greatest tendency towards hair formation, and are also one of the most widespread cyanobacterial families.

1.3 HAIRS IN EUKARYOTIC ALGAE

A brief review of the occurrence of hairs in eukaryotic algae will be considered as a comparison to the presence of hairs in cyanobacteria. Hairs in members of the green, brown and red algae are well known to phycologists (Whitton, 1988). These hairs are easily recognizable and have an important taxonomic function. The hairs are divided into a multicellular hair, unicellular hair and a prolongation from a vegetative cell known as a seta

(Whitton, 1988). The occurrence of different types of hair in eukaryotic algae are summarized below (Whitton, 1988). In Chapter 8 a brief study of the localization of phosphatase activity in eukaryotic hairs will be reported.

Table 1.2 The occurrence of various types of hair in eukaryotic algae.

Taxonomy	Feature of hair
CHLOROPHYTA	
Chaetophorales	
Chaetophoraceae	Multicellular, usually showing decrease or loss of chloroplast on passing towards apex and often (e.g. Stigeoclonium) tapered from base to apex.
Aphanochaeteceae	1-cellular, simple, no chloroplast, sometimes bulbous at base, no sheath; detailed morphology is diagnostic feature for many genera.
Chaetosphaeridiaceae	Probably always a cytoplasmic process (seta), but ultrastructural studies needed: very narrow, often single or double sheath at base, sometimes with several (e.g. Conochaete) setae arising from one basal cell.
Coleochaetaceae	Cytoplasmic process (seta), with sheath at base; extension of chloroplast present in seta.
Dicranochaetaceae	Probably cytoplasmic process (seta), but ultrastructural studies needed; branched.
Oedogoniales	
Bulbochaete	1-cellular, no chloroplast, bulbous base.
Caulerpales	1-cellular hairs on some species of Codium.
Dasycladales	Acetabularia has whorls of branching hairs arising from main axis below cap.
PHAEOPHYTA	Hairs are present in all orders, with examples from many genera or species.
Ectocarpales	Multicellular, typically with chloroplasts showing decreasing prominence from base to apex (e.g. Pleurocladia).
Sphacelariales	Multicellular, no chloroplast, sheathed at base (e.g. some Sphacelaria spp.).
RHODOPHYTA	Common among Florideae (see Fritsch, 1945): mostly 1-cellular, narrow and often very long, no chloroplast, but often (always?) with plug of cytoplasm at tip (e.g. Lemanea), which at least in Gelidium caulacantheum contains a nucleus.

1.4 POSSIBLE FUNCTIONS OF HAIRS

Most of the literature has ascribed possible functions to eukaryotic hairs and these roles could be used for cyanobacterial hairs. Previously it was suggested that hairs may function as a light screen, although DeBoer and Whoriskey (1983) showed that light had no effect on abundance, length or distribution of hairs in Ceramium rubrum. Drumgole and Booth (1985) suggested that hairs in Gelidium caulacantheum might aid in water retention, and that the water holding capacity of the hair zone may be as much as 300% more than the internal water content of the thallus.

The frequent finding that hairs are associated with nutrient deficiency has led most authors to speculate that hairs are associated with nutrient uptake (Whitton, 1988). Gibor (1973) has provided direct evidence for hairs playing a role in uptake of vital dyes (neutral red and methylene blue) in Acetabularia and that the uptake was under metabolic control.

The most convincing evidence for hairs specializing in nutrient uptake is supplied by DeBoer and Whoriskey (1983) for Ceramium rubrum. A two component model can be used to fit ammonium uptake by C. rubrum at external NH_4-N concentrations below 40 µM. At concentrations less than 10 µM, a high affinity uptake system (component one) predominates and at concentration between 10 - 40 µM a strong "diffusive" system (component two) predominates; this "diffusive" component may be a system with a high K - half saturation values and high V_{max} - maximum rates of uptake (D'Elia and DeBoer, 1978). Component one uptake was independent of the hairs, whereas component two uptake was markedly enhanced by the presence of hairs, suggesting separate sites for components one and two. The authors postulated that hairs function primarily by increasing the uptake sites for component two and that they are adapted to taking full advantage of intermittent bursts of high concentrations of nutrients.

Sinclair and Whitton (1977a) demonstrated that hair formation in 13 Rivulariaceae strains was stimulated by phosphate deficiency. Livingstone et al., (1983) demonstrated that phosphomonoesterase activity (1.61) commenced at the same time as hair formation in Calothrix parietina D550. Phosphomonoesterase activity was localized on the hair cells, vegetative cells adjacent to the hair cells and on the sheath furthest from the heterocyst. Phosphatase activity may be very important to this alga in nature, as it was isolated from a stream where most of the soluble phosphate is organic (Livingstone and Whitton, 1984). Rivularia colonies taken from this stream and other similar environments also showed marked phosphatase activity and phosphate uptake from β-glycerophosphate was 86% of that from inorganic phosphate. Further indication that hairs may be sites of phosphatase activity in the Chlorophyta was demonstrated by Gibson and Whitton (1987) for phosphate deficient strains of Chaetophoraceae. Hair formation commenced when the P level had fallen below 1% dry weight and phosphatase activity was localized on the hairs using the lead capture technique.

The most likely role of hairs in the Rivulariaceae seems to be as sites of phosphatase activity and phosphate uptake, which will possibly be related to the P status of the environment. Therefore, the study concentrated on the relationship between trichome morphology, physiology, phosphatase activity and phosphate uptake in the Rivulariaceae.

1.5 PHOSPHORUS OCCURRENCE AND FORMS IN THE ENVIRONMENT

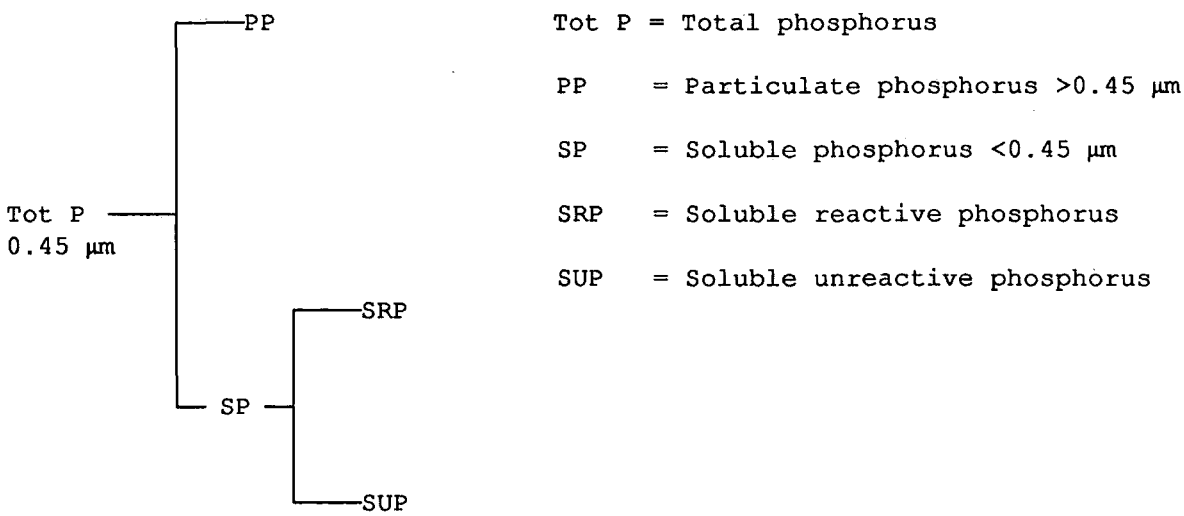
Phosphorus (P) is the eleventh most abundant element in nature. Its concentration is estimated as 0.1% by weight in the lithosphere and is thus classed as a trace element. 80% of the P reserves are contained in phosphorite deposits in ocean sediments and 15% in igneous and metamorphic rocks. Phosphorus is a necessary plant nutrient and is defined as a rate limiting element.

Phosphorus generally occurs in the oxidized form, either as phosphates or organic P compounds. Phosphates can be divided into:

1. Orthophosphates
2. Polyphosphates (chain phosphates)
3. Metaphosphates (ring phosphates)
4. Ultraphosphates (branched ring phosphates)

Orthophosphates are generated from the weathering of rocks or from biological metabolism or degradation. Polyphosphates and metaphosphates are produced by biological activity. Orthophosphates and polyphosphates are frequently introduced into waters by man (Broberg and Persson, 1988). Analytically defined P fractions are categorized in the following manner:

Table 1.3 Analytically defined phosphorus fractions (Rigler, 1973).



Particulate P (colloidal P)is derived from five sources:

1. Cells of plants, bacteria and animals.
2. Weathering products such as primary or secondary minerals.
3. Direct precipitation of inorganic P or adsorption onto other precipitates.
4. Degradation and fragmentation of cells, providing organic detritus.
5. Flocculation of organic macromolecules, resulting in larger sized aggregates.

Particulate organic P in aquatic ecosystems dominates total organic P, and is comprehensively reviewed by Broberg and Persson (1988). Although particulate organic P is the major constituent of organic P forms it is not readily available. Dissolved organic P (DOP) can be regarded as a more important fraction as it is readily available to the biota and is rapidly turned over. It is not known whether DOP is primarily released by active cell metabolism or by cell death and decay.

Some pools of DOP do not undergo rapid hydrolysis by phosphatases, and these compounds may constitute a major part of the DOP pool. One such pool of DOP compounds are the nucleotides or polynucleotides. Phillips (1964) obtained six DOP fractions from sea water, three of which were identified as nucleotides or polynucleotides. Broberg and Persson (1988) reported that up to 4.2% of the total P in bogs was attributed to nucleic acid. Minear (1972) found that up to 50% of high molecular weight DOP excreted from organisms was DNA or its fragments. Hino (1989) found that 65% of DOP in lake waters was composed of compounds between 300 to 10000 daltons. Addition of phosphodiesterases (1.612) to high molecular weight DOP compounds did not release any measurable P_i, although a combination of phosphodiesterases and phosphomonoesterases increased the amount of P_i released by 30% when compared to release of P_i by PMEases alone.

A major component of colloidal P in aquatic systems are the inositol phosphates or phytates, which are hydrolyzed by the group of phosphatases known as phytases (Mitchell and Read, 1981). The phytates are esters of inositol and phosphoric acid. Phytates exist in many forms as there are many different isomers of inositol and each isomer exists with one to six esterified phosphate groups per molecule. Phytates can comprise up to 35% of the colloidal P, which is mainly derived from microbial storage and structural compounds.

1.6 PHOSPHATASES

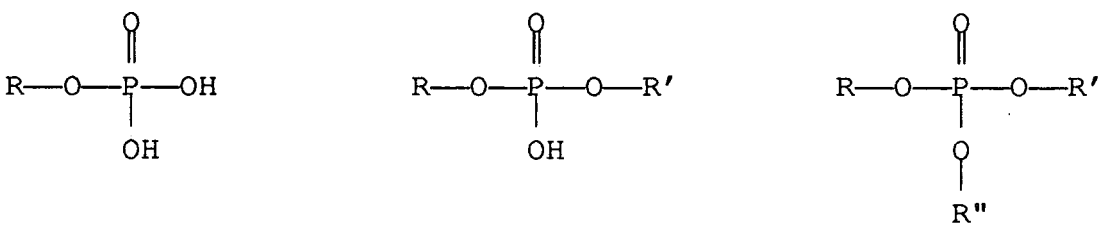
1.61 INTRODUCTION

Phosphatases are enzymes which promote the degradation of a wide variety of complex P compounds into orthophosphate (P_i) and an organic moiety (Jansson et al., 1988). Phosphatases are thought to have an essential function in the nutrient dynamics of the environment.

1.611 Phosphomonoesterase

Most often the term "phosphatase" is used synonymously with phosphomonoesterase, abbreviated to PMEase (Flynn et al., 1986). Similar but functionally different enzymes are the phosphodiesterases, abbreviated to PDEase, which include the nucleases. General formulae for phosphate esters are in Fig. 1.1 (below).

Fig. 1.1 Formulae for phosphate esters.



PHOSPHOMONOESTER PHOSPHODIESTER PHOSPHOTRIESTER

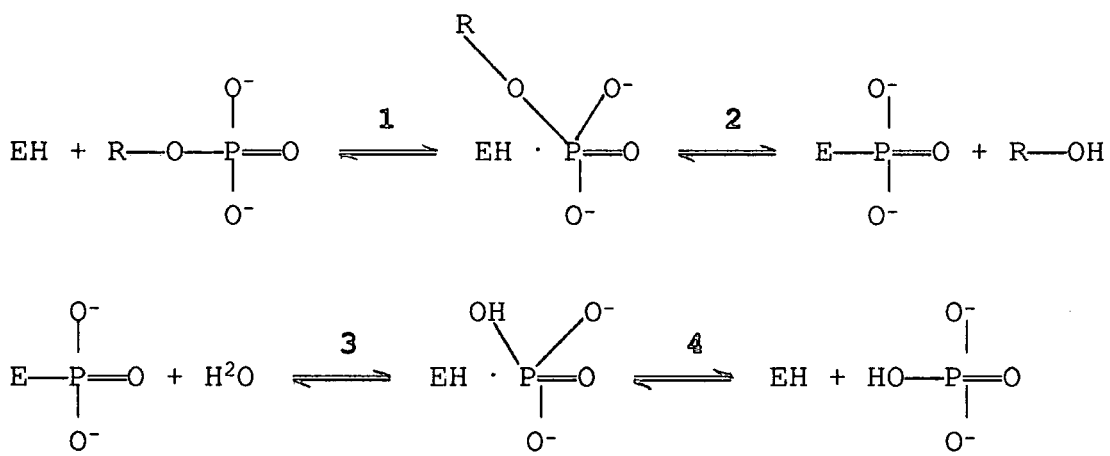
R represents the organic part of the phosphate esters.

The most common catalytic breakdown studied is the breakdown of phosphomonoesters by PMEases. The reaction mechanism (Fig. 1.2) is divided into four steps (McComb et al., 1979):

1. Non-covalent binding of the substrate to the enzyme (EH).
2. Alcohol release from the complex and P_i becomes covalently bound to the enzyme forming a phosphoryl-enzyme compound.
3. Conversion of the phosphoryl-enzyme compound, through uptake of water, to a non-covalent complex.
4. Release of P_i and regeneration of free enzyme.

Any of the steps 2-3 can be rate limiting for the overall reaction (McComb et al., 1979).

Fig. 1.2 Reaction scheme for the enzyme catalyzed P of phosphate esters as described by McComb et al. (1979).



PMEase activity will primarily depend on the type and concentration of substrate and enzyme. Other factors which affect PMEase activity are temperature, ionic strength, pH and metal ions (McComb et al., 1979). Alkaline PMEases have been characterized as metallo-enzymes with an essential

metal ion, which has been reported to be zinc in many cases (Spiro, 1973; McComb et al., 1979 and Torriani-Gorrini et al., 1987).

1.612 Phosphodiesterases

PDEases or nucleases have been isolated in a wide range of organisms (Razzel and Khorana, 1959) e.g. in snake venom (Razzel and Khorana, 1959), hog (Razzel, 1959), cow (Kelly et al., 1975), rat (Futai and Mizuno, 1967), carrot (Harvey et al., 1970) and in human urine (Ito et al., 1987). PDEases are able to hydrolyze a wide range of nucleotides. PDEases are distinguished into two categories on their ability to hydrolyze 3′ and 5′ nucleotides. Phosphodiesterase I hydrolyzes nucleic acids to nucleoside 5′-phosphates and phosphodiesterase II hydrolyzes nucleic acids to nucleoside 3′-phosphates (Kelly et al., 1975). The ability of PDEases to hydrolyze nucleic acids is incorporated as a tool in molecular biology.

PDEases are typically alkaline and inhibited by EDTA. The activity of EDTA treated PDEases is completely restored by zinc and partially by calcium and magnesium (Ito et al., 1987), which suggests that PDEases are also zinc-metallo enzymes like PMEases. However the amino acid composition of PDEases differs markedly from PMEases localized from the same tissue (Kelly et al., 1975). PDEases in all cases are inhibited by P_i and in most cases PDEases are inhibited by ascorbic acid. It has been demonstrated that preparations of supposedly purified PMEases have diesterase activity. This activity was attributed to contaminating PDEases.

1.62 Bacterial phosphatase activity

A large amount of work has concentrated on PMEases in heterotrophic bacteria, with most research on PMEase structure and function in E. coli (McComb et al., 1979). PMEase activity in freshwater heterotrophic bacteria has been demonstrated in lake waters (Reichardt et al., 1967; Jones, 1972,a; Pettersson, 1980; Chrost et al., 1984; Hallemejko and Chrost, 1984). The

majority of work on aquatic bacterial PMEases has been carried out with marine forms. Marine bacteria, in general, appear to have PMEases that are located in the periplasmic space (Thompson and MacLeod, 1974a, b). The production of extracellular PMEases in heterotrophic bacteria is less common than in algae (Jansson et al., 1988).

1.63 Algal phosphatase activity

Cell-bound PMEase activity in algae is widespread, though not universal (Healey, 1982). PMEase activity has been demonstrated for many genera of cultured algae (Kuenzler, 1965; Kuenzler and Perras, 1965; Healey, 1973; Healey and Hendzel, 1975; Flynn et al., 1986; Doonan and Jensen, 1980; Wynne, 1981; Smith and Kalff, 1981). PMEases have been located on the cell surface (Brandes and Elston, 1956); in cell membranes (Kuenzler and Perras, 1965; Møller et al., 1975; Flynn et al., 1986) and extracellular enzymes in cultured algae are frequently reported (Aaronson, 1971; Healey, 1973; Aaronson and Patni, 1976; Patni et al., 1977; Healey and Hendzel, 1979; Wynne, 1981).

The synthesis of PMEases in algae is influenced mainly by substrate supply and reaction products. In most cases algal PMEases are inducible and activity increases with the onset of P-deficiency, although no significant increases in PMEase activity were detected in P-deficient Synechococcus (Kuenzler, 1965), Coccochloris (Kuenzler and Perras, 1965) and Pseudanabaena catenata (Healey and Hendzel, 1979). In these algae the PMEases are probably constitutive, i.e. they are more or less constantly synthesized in the cell.

PMEase activities in aquatic environments have often been attributed to algae (Heath and Cooke, 1975; Pettersson, 1980). The major amount of work on activity in aquatic environments has concentrated on extracellular PMEases (Jansson et al., 1988), which can make up a substantial amount of the activity in lake waters. Extracellular PMEases are generally defined as those which pass through 0.45 μm membrane filters. The characteristics of extracellular

PMEases do not differ from "cell-bound" PMEases (Glew and Heath, 1971 and Flynn et al., 1986). It is not clear how, or to what extent, active secretion of PMEases takes place and whether it is more beneficial to release PMEases or to localize them on the external cell surface (Jansson et al., 1988).

1.64 Characteristics of algal phosphatases

1.641 Acid and alkaline

Optimum PMEase activity occurs at different pH values and hence the common separation into acid and alkaline PMEases. Acid PMEases generally have the highest activity between pH 4 - 6 and alkaline PMEases between pH 8 - 11. Both alkaline and acid PMEases have been found as extracellular and cell-bound enzymes in algae (Siuda, 1984).

Acid and alkaline PMEases are similar in that they have a broad specificity against different substrates, i.e. their activity is only restricted to the P-O bond on the phosphomonoesters. However, alkaline PMEases differ in that they require cations for activity and are inhibited by chelators such as EDTA (Whitt and Savage, 1988), whereas acid PMEases have no cationic requirement and are specifically inhibited by fluoride (Cembella et al., 1984a).

Jansson et al. (1981) found four different acid PMEases, based on their molecular weights, in Lake Gårdsjön. They were all inhibited by P_i and had K_m values similar to alkaline PMEases. It was proposed that these PMEases had adapted to acid conditions and had the same functions as typical alkaline PMEases from lakes with a higher pH.

Acid and alkaline PMEases have an essential difference concerning their location in the cell and mode of synthesis. Acid PMEases are intra-cellular (cellular) whereas alkaline PMEases are in contact with the surrounding medium i.e. bound to the cell membrane, wall or sheath (Møller et al., 1975; Wynne,

1977; Schmitter and Jurkiewicz, 1981; Siuda, 1984). In contrast to alkaline PMEases acid PMEase synthesis is generally not inhibited by P_i (Wynne, 1977). It has therefore been hypothesized that acid PMEases are constitutive enzymes produced for internal P-metabolism (cellular PMEases), whilst alkaline PMEases have external functions and a synthesis which is induced or repressed depending upon the P status of the alga (Jansson et al., 1988).

1.642 Temperature dependence

Algal PMEases have Q_{10} values between 1.5 and 3 and temperature optima between 30 - 60°C, which is usually above the temperature of the original environment. Huber and Kidby (1984) showed that temperature optima of algal cultures and field populations were between 25 - 50°C.

1.643 Effect of ions

The ionic requirements for algal PMEases vary considerably. Calcium has the greatest stimulatory effect on algal PMEases (Glew and Heath, 1971; Healey, 1973; Doonan and Jensen, 1980). Glew and Heath (1971) determined that there were 8 g atoms of Ca^{++}/ mole of PMEase in Micrococcus sodenensis. Magnesium has little or no effect on algal PMEases, although it has been reported to have a stimulatory effect on a wide range of heterotrophic bacterial PMEases (Schlesinger et al ., 1969; Day and Ingram, 1973; Thompson and MacLeod, 1974a).

Zinc inhibited alkaline PMEase activity in Plectonema boryanum (Doonan and Jensen, 1980) and Anacystis nidulans (Ihlenfeldt and Gibson, 1975). Walther and Fries (1976) showed enhancement of extracellular alkaline PMEase activity by Zn^{++} in a multicellular marine alga. Zn^{++} enhanced activity in a wide range of heterotrophic bacteria in direct contrast to its effect on cyanobacteria (Schlesinger et al., 1969; Sakaguchi et al., 1972; Day and Ingram, 1973).

Manganese and cobalt slightly enhanced activity in Plectonema boryanum, Anacystis nidulans and Vibrio parahaemolyticus (Sakaguchi et al., 1972; Ihlenfeldt and Gibson, 1975; Doonan and Jensen, 1980). Sodium and potassium had a negligible effect on PMEase activity. P_i inhibited most inducible alkaline PMEase systems (Healey, 1973; Ingram et al., 1973; Ihlenfeldt and Gibson, 1975; Doonan and Jensen, 1980). EDTA inhibits alkaline PMEase activity (Whitt and Savage, 1988), which suggests the importance of one or more metals in algal PMEases, although no algal PMEases have been isolated to confirm this.

1.644 Substrate affinity

The ability for PMEases to hydrolyze substrates is given by the K_m value (Michaelis-Menten constant). K_m is the substrate concentration when the reaction proceeds at half its maximum speed. A low K_m means that the enzyme has a high affinity to the substrate and the opposite for a high K_m. Therefore, the use of the Michaelis-Menten equation for a mixture of enzymes, which has been carried on algal PMEases, is theoretically incorrect. However, from a practical standpoint, a group of enzymes can be characterized by solving a Michaelis-Menten equation.

K_m varies with substrate structure, indicating that PMEases are not substrate specific (Jansson et al., 1988). Other factors which significantly affect K_m are pH and temperature. Usually the K_m values reported are between 10^{-6} to 10^{-4} M for acid and alkaline PMEases.

Pettersson (1980) demonstrated that the K_m varied annually by an order of magnitude in Lake Erken, with the lowest values during periods of extreme P-deficiency. He hypothesized that phytoplankton adapted to P-deficiency by increasing enzyme production and producing enzymes with an improved ability to utilize lower substrate concentrations.

1.645 Stability

Extracellular PMEases are functional for long periods under axenic conditions. Alkaline PMEases when incubated with chloroform-saturated water, decreased in activity by 20% over 10 d (Berman, 1970). Jansson et al., (1981) found that extracellular acid PMEases remained active after 20 d and 10% of the original activity remained after 69 d. Halemejko and Chrost (1984) reported that extracellular PMEase activity in lake waters remained unaffected after 4 d. However, the mechanism for the inactivation or breakdown of phosphatases in situ remains unclear.

1.646 Control of synthesis

Inducible PMEases are those where synthesis starts in the presence of suitable substrates, and constitutive PMEases are enzymes produced independently of an activator, i.e. they are more or less constantly synthesized in the cell. Induction, where PMEase activity is enhanced by the addition of a substrate, seems uncommon or rarely investigated. Aaronson and Patni (1976) demonstrated that the secretion of acid PMEases in Ochromonas danica increased after addition of glucose-1-phosphate and glucose-6-phosphate.

Algal acid PMEases are generally constitutive whilst alkaline PMEases are inducible. Acid PMEases are located internally, whilst alkaline PMEases have external functions (1.341). Therefore, alkaline PMEases supply algae with P_i from outside the cell and are regulated by the internal P_i pool (Fitzgerald and Nelson, 1966; Wynne, 1977; Pettersson, 1980, 1985 and Livingstone et al., 1983). When the internal P_i pool is filled synthesis of alkaline PMEases is stopped, and when the pool is depleted to a particular level, alkaline PMEase synthesis is induced.

1.65 Alkaline phosphatase activity as a phosphorus-deficiency indicator

Kuenzler and Perras (1965) and Fitzgerald and Nelson (1966) stated that P-limited marine and freshwater algae produced alkaline PMEases and the production stopped when the algae were P-sufficient. These two papers were the basis for the use of algal alkaline PMEases as biological indicators of the P-status of the environment. Healey (1982) confirmed that many algal species respond in a similar manner to P-deficiency that it was possible to measure PMEase activity in natural mixed populations.

The potential PMEase activity and its variation has been used by several investigators as an indicator of P-deficiency. These investigations demonstrated an inverse relationship between alkaline PMEase activity and the following variables (Jansson et al., 1988):

1. Cell-bound alkaline PMEase activity and P_i concentration (Pettersson, 1980, 1985; Francko, 1984; Chrost et al., 1984).
2. Extracellular alkaline PMEase activity and P_i concentration (Reichardt, 1971).
3. Alkaline PMEase activity and total P concentration (Berman, 1970; Smith and Kalff, 1981).
4. Alkaline PMEase activity and total cellular surplus P (Pettersson, 1980, 1985).
5. Cellular alkaline PMEase activity and total cellular P (Wynne, 1977).

In each case low alkaline PMEase activity was associated with high concentrations of P. High alkaline PMEase activity was only detected when the P concentration was low.

PMEase activity is always related to biomass of the PMEase producing organisms in the assay, giving the specific potential PMEase activity. Several biomass estimators have been used, dry weight (dry wt), particulate carbon, adenosine triphosphate (ATP) and chlorophyll a (Pettersson, 1980). Biomass estimators such as chlorophyll a can vary due to interspecific or

environmental variations. In situations where there are large amounts of detritus, ATP may be preferable in order to estimate activity from the living biomass (Pettersson, 1980). Healey (1982) quoted that the specific level of alkaline PMEase activity for P-deficient cyanobacterial cultures is above 2 μmol P hydrolyzed (≡ 2 μmol ρNP) mg dry wt^{-1} h^{-1}.

Induction of alkaline PMEase activity is often associated with low total cellular P concentration, low polyphosphate concentration and high polyphosphate synthetase activity (Healey, 1982). Therefore, alkaline PMEase activity, total cellular P, polyphosphate concentration and polyphosphate synthetase activity are in themselves P-deficiency indicators. However, they are not independent of each other since repression-induction of alkaline PMEase activity is controlled by cellular P fractions, probably polyphosphate (Cembella et al., 1984a).

Olsen et al. (1983) showed that induction of the synthesis of alkaline PMEase in Chlamydomonas reinhardtii started below an internal P level of 3 - 3.5 μg P mg dry wt^{-1}. Fitzgerald and Nelson (1966) concluded that a surplus P (polyphosphate) level of 0.8 μg P mg dry wt^{-1} or lower indicated a likelihood for induction of phosphatase synthesis. This observation was confirmed by Healey (1973) for Anabaena variabilis. Pettersson (1980, 1985) reported values between 0.2 - 1.0 μg P mg dry wt^{-1} for spring phytoplankton in Lake Erken with high alkaline PMEase activity and a threshold value of 0.5 μg P mg dry wt^{-1}.

Cembella et al. (1984a) argued that "the current practice of using assays of alkaline PMEase as bio-indicators of the nutritional status of the environment is probably reckless and fraught with undesirable complications". They agreed that PMEases from zooplankton (Boavida and Heath, 1984), bacteria, degenerating cells, external input of PMEases (Stevens and Parr, 1977), dissolved PMEases (Jansson et al., 1981) and constitutive PMEases (Fitzgerald and Nelson, 1966) could all severely decrease the significance of the assay as

an indicator of P-deficiency. Increased PMEase activity can also be induced by factors other than P-deficiency. Wilkins (1972) showed that alkaline PMEase activity in E. coli was induced by a deficiency in pyrimidines and guanine and not a lowering of the internal P_i pool. Francko (1984) showed that increases of 525% or decreases of 58% in alkaline PMEase activity can be induced by the addition of 0.2 - 10 nmol l^{-1} of cyclic adenosine monophosphate (cAMP). Francko and Wetzel (1982) showed that cAMP was in the range 16 - 324 pmol l^{-1} in freshwater environments. Variations in PMEase activity during the day have also been demonstrated (Reichardt, 1971; Wynne, 1981; Chrost et al., 1984; Huber and Hamel, 1985). This stresses the problem that even the time of sampling is a factor influencing PMEase activity.

1.66 Measurement of phosphatase activity

Phosphatase activity is assayed by hydrolysis of a suitable artificial substrate and the subsequent detection of the increase over time of an organic product or orthophosphate. The substrate concentration used is high enough so that the reaction proceeds at maximum velocity for a reasonable length of time.

Phosphatase activity can be separated into many different fractions. Phosphatase activity can be measured as the total without pre-treatment of the sample, which represents extracellular and cell-bound phosphatases. Differential filtration determines activity associated with varying sizes of particles. The activity can be measured in the filtrate giving the particulate activity as the difference between total and extracellular activity. Analysis of cellular phosphatases is achieved after sonical (Reichardt, 1971; Rivkin and Swift, 1980; Klotz, 1985), mechanical (Lin, 1977), chemical (McComb et al., 1979 and Marco and Orús, 1988) or enzymatic (Ihlenfeldt and Gibson, 1975) rupturing of the cell contents. However the release of cellular phosphatases into a new environment may result in

inactivation of enzyme activity (Talpasayi, 1962). Phosphatase activity measured routinely in the laboratory cannot be used for predictions of *in situ* hydrolytic activity. This is because:

1. Substrate concentrations are lower in nature than those used in routine phosphatase assays.
2. Standardized temperature and pH are often not representative of the original environment.
3. The structure of natural substrates is unknown. Although activity is restricted to the P--O bond of phosphate esters, the affinity to the substrate can vary depending on the structure of the organic moiety.

The six most common substrates used to detect activity are methylumbelliferyl phosphate (MUP), methylfluorescein phosphate (MFP), ρ-nitrophenyl phosphate (ρNPP), glycerophosphate, phenolphthalein phosphate and 1-naphthol phosphate (Pettersson and Jansson, 1978). The most sensitive substrates are the fluorogenic compounds MUP and MFP (Perry, 1972; Jansson, 1976, 1977; Kobori and Taga, 1979; Healey and Hendzel, 1979, 1980; Smith and Kalff, 1981; Francko, 1983, 1984; Currie and Kalff, 1984; Bothwell, 1985). The problem with MFP is that it has a high background fluorescence and a lower MFP concentration is needed to reduce this interference. The most common substrate used is ρNPP, where the product ρ-nitrophenol (ρNP) is detected colorimetrically between 400 - 415 nm (Reichhardt *et al.*, 1967; Berman, 1969, 1970; Reichardt, 1971; Jones, 1972a,b; Heath and Cooke, 1975; Stevens and Parr, 1977; Wynne, 1977,1981; Doonan and Jensen, 1980; Tiwari and Mishra, 1982; Livingstone *et al.*, 1983; Livingstone and Whitton, 1984; Chrost *et al.*, 1984, Klotz, 1985a,b). ρNPP is suitable for long incubations or where there is high activity and fluorogenic substrates are superior for assays where activity is low.

It must be stressed that PMEase activity is dependent on the substrate used, eg ρNPP is easily hydrolyzed by PMEases and may give misleading representation of the naturally occurring hydrolytic activity.

1.7 PHOSPHATE UPTAKE

Phosphatases hydrolyze phosphomonoesters into P_i and an organic moiety. Combined with the ability to rapidly hydrolyze a wide range of phosphomonoesters efficient consumers of organic P, such as the Rivulariaceae under study, must have an efficient uptake system to utilize the P_i released. For this reason a brief overview of the status of knowledge of P_i uptake in bacteria is included.

1.71 Phosphate uptake in bacteria

Phosphate uptake in heterotrophic bacteria has been studied intensively with most of the work concentrating on E. coli. The principal features of P_i uptake were described by Medveszky and Rosenberg (1971) who confirmed the presence of a high and low affinity uptake system. The high affinity system was activated only when the internal P_i pool was depleted. The high affinity system functioned simultaneously with the low affinity system to rapidly fill the P_i pool. Once the pool was filled only the low affinity system remained active. The low affinity system would supply P_i at the rate at which it was being metabolized. The internal P_i pool had a defined capacity and all P_i entering the cell passed this pool before utilization.

In a series of investigations (Medveszky and Rosenberg, 1971; Gerdez and Rosenberg, 1974; Gerdes et al., 1977) a membrane bound protein was found to be associated with the high affinity system. This protein had a molecular weight of 42000, and one molecule of protein bound one molecule of P_i. It was possible to restore P transport in P-deficient cells by addition of the

protein alone. During P-deficiency the concentration of this protein increased 100 fold (Willsky and Malamy, 1976).

The genetic regulation of the two transport systems was characterized by Willsky et al. (1973) and they termed them Pit (phosphate inorganic transport) and Pst (phosphate specific transport). The Pit corresponded to low affinity uptake and Pst to high affinity uptake. These terms are now generally accepted.

Willsky and Malamy (1980) described kinetically the two systems. Pit was a low affinity, high velocity system (high K_m and high V_{max}) and the Pst system was a high affinity, low velocity system (low K_m and low V_{max}). This implied that Pst is adapted to transport P_i at low external concentrations and the Pit system to high P_i concentrations. Willsky and Mallamy (1980) also found that growth in high P_i medium led to Pit contributing 90% and Pst 10% to uptake.

Pst is inducible and repressible by P_i and Pit is constitutive (Rosenberg et al., 1978). The energy source for Pst is suggested to be ATP, whilst Pit is thought to be driven by the proton motive force (Rosenberg et al., 1979). A close connection between P_i uptake and K^+ was reported by Russel and Rosenberg (1979), who stated that P_i and K^+ were taken up simultaneously. When the internal pool is saturated with P_i, uptake is not switched off, instead it continuously exchanges P_i between the external medium and the internal pool of P_i (Medveszky and Rosenberg, 1971).

1.72 Phosphate uptake in algae

A number of investigations have measured P_i uptake in algae, but have failed to describe critically the mechanisms involved. Healey (1982) stated V_{max} values of PO_4 uptake in P-deficient cyanobacteria between 0.6 μmol P mg^{-1} h^{-1} in Plectonema boryanum to 1.8 μmol P mg^{-1} h^{-1} in Anabaena flos-aquae. In algae monophasic uptake systems predominate (Perry, 1976; Chisholm and Stross, 1976a,b; Nyholm, 1977; Tilman and Kilham, 1976). However multiphasic

uptake systems have been described. JeanJean (1976) suggested a high and low affinity system in Chlorella pyrenoidosa after studying the effects of metabolic inhibitors on P_i uptake. The systems described were very similar to E. coli although no transport protein was found.

Two affinity systems were described for Euglena gracilis (Chisholm and Stross, 1976a,b) and triphasic systems were reported for Pyrocystis noticula (Rivkin and Swift, 1982) and Anacystis nidulans (Simonis et al.,1974). However these investigations are insufficient for suggesting that multiphasic uptake systems are general in algae.

The lack of multiphasic algal uptake could reflect improper experimentation as uptake kinetics can be influenced by many variables:

1. The ratio of cell number to P_i concentration i.e. P_i availability per cell.
2. The physiological status of the algae.
3. The concentration of cations in the medium, Mg^{++} (Healey, 1973) and Ca^{++} (Falkner et al., 1976), affect P_i uptake.
4. Between pH 7.5 - 8.5 uptake is optimal for cyanobacteria, suggesting that HPO^{2-} is the major species taken up.

P-deficient algae take up P faster than P-sufficient algae. Uptake rates can increase up to 100 fold in P-deficient cells (Rhee, 1973; Brown et al., 1978; Brown and Button, 1979). It appears this response is controlled by the internal P_i pool, particularly the polyphosphates (Blum, 1966; Healey, 1973). The polyphosphate pool is common in many algae (Cembella et al., 1984b) and is filled during periods of high P supply. Storage of P as polyphosphate can continue until the P content of the cells greatly exceeds that of exponentially growing cells (Stewart et al., 1978). The rapid formation of polyphosphate is mediated by the enzyme polyphosphate synthetase, which increases during P-deficiency (Grillo and Gibson, 1979). The deposition of polyphosphate is also dependent on calcium, which is deposited along with polyphosphate (Stewart et al., 1978). This phenomenon of excessive storage

of P is known as luxury consumption (Healey, 1982). The polyphosphate pool can sustain growth for several generations in the absence of external P.

The content of the polyphosphate pool is a reflection of the external P supply and polyphosphate content has been used as a P-deficiency indicator (Fitzgerald and Nelson, 1966). Rhee (1973) studied the role of polyphosphates in algae and suggested that one fraction (polyphosphate A) served as a non-competitive inhibitor in the transport system.

1.8 AIMS

Sinclair (1977) concluded that hairs in the Rivulariaceae were a response to P-deficiency, although no specific role was ascribed to them. The overall emphasis of the research was to discover if hairs in the Rivulariaceae were the major sites of phosphatase activity and phosphate uptake, and if so were hair cells specifically adapted to utilize high concentrations of organic P sources in the environment.

The initial aim was to survey all axenic Rivulariaceae isolates in the Durham Culture Collection and some non-Rivulariaceae isolates for comparison, concentrating on P-deficiency, possible associated phosphatase activities and hair formation. This would determine if there were major patterns or differences within the Rivulariaceae. Further work concentrating on hair-forming Rivulariaceae strains would determine the role of trichome structure and hair cells.

A further aim was to establish the relationship between the presence or absence of hairs and the nutrient status and or cycling of nutrients in the environment, i.e. are hair-forming strains associated or adapted to a particular environment?

Most studies would concentrate on laboratory isolates, although localization of phosphatase activity in a wide range of hair-forming algae would include field material.

CHAPTER 2

MATERIALS AND METHODS

2.1 COMPUTING

Three computer systems were used during this study. Routine calculations were performed on a Research Machines Nimbus XN16 using the MULTIPLAN spreadsheet program (Microsoft). Statistical analyses were carried out using SPSSX running on an AMDAHL 470/V8 mainframe operating under the Michigan Terminal System. Graphical output was carried out using the UNIRAS suite of software, incorporating UNIGRAPH and UNIEDIT, running on a SUN minicomputer operating under UNIX.

2.11 Durham Culture Collection

All unialgal strains in the Durham Culture Collection are assigned a unique three digit number. The Durham Culture Collection records are held on a Research Machines Nimbus XN16 operating under MS-DOS; the database software used was Superfile (Southdata, London).

2.2 CYANOBACTERIA USED IN THE STUDY

A list of the 51 cyanobacterial strains used in the study is presented in Table 2.1 along with their origins, habitats, taxon and the presence or absence of hairs.

Table 2.1 List of the 51 Cyanobacteria used in the study.

No.	Location	Genus	Taxon	Habitat	Env	Hair
750	Thailand	Anabaena	N	deepwater rice	NO	-
669	North America	Anabaena	N	pond	NO	-
217	Aldabra	Anabaena	N	pond	CA	-
746	Thailand	Anabaena	N	deepwater rice	NO	-
255	North America	Calothrix	Y	pond	NO	-
251	North America	Calothrix	Y	stream	NO	+
794	Nepal	Calothrix	Y	paddy rice	NO	-
182	India	Calothrix	Y	paddy rice	NO	-
184	England	Calothrix	Y	stream	CA	+
796	Nepal	Calothrix	Y	paddy rice	NO	-
253	Cuba	Calothrix	Y	marine	MAR	+
624	Bangladesh	Calothrix	Y	deepwater rice	NO	-

No.	Location	Genus	Taxon	Habitat	Env	Hair
764	Bangladesh	Calothrix	Y	deepwater rice	NO	-
550	England	Calothrix	Y	stream	CA	+
802	Bangladesh	Calothrix	Y	deepwater rice	NO	-
254	Shri Lanka	Calothrix	Y	pond	NO	-
795	Nepal	Calothrix	Y	paddy rice	NO	-
690	Saudi Arabia	Calothrix	Y	stream	CA	+
730	Thailand	Calothrix	Y	deepwater rice	NO	-
266	France	Calothrix	Y	stream	HM	+
786	India	Calothrix	Y	soil	NO	-
603	Bangladesh	Calothrix	Y	deepwater rice	NO	-
688	Saudi Arabia	Calothrix	Y	stream	CA	-
202	Aldabra	Calothrix	Y	pond	CA	-
694	Thailand	Calothrix	Y	paddy rice	NO	-
684	West Germany	Cylindrospermum	N	pond	NO	-
809	New Zealand	Dichothrix	Y	stream	NO	+
808	Albania	Dichothrix	Y	stream	CA	+
689	Saudi Arabia	Dichothrix	Y	stream	CA	-
612	Bangladesh	Fischerella	N	deepwater rice	NO	-
572	Philippines	Gloeotrichia	Y	paddy rice	NO	+
626	Bangladesh	Gloeotrichia	Y	deepwater rice	NO	-
743	Thailand	Gloeotrichia	Y	deepwater rice	NO	-
281	North America	Gloeotrichia	Y	pond	NO	-
602	Bangladesh	Gloeotrichia	Y	deepwater rice	NO	-
613	Bangladesh	Gloeotrichia	Y	deepwater rice	NO	-
627	Bangladesh	Lyngbya	N	deepwater rice	NO	-
608	Bangladesh	Nostoc	N	deepwater rice	NO	-
611	Bangladesh	Nostoc	N	deepwater rice	NO	-
734	Thailand	Nostoc	N	deepwater rice	NO	-
614	Bangladesh	Nostoc	N	deepwater rice	NO	-
800	England	Nostoc	N	pond	NO	-
201	Aldabra	Nostoc	N	pond	CA	-
767	Bangladesh	Synechococcus	S	deepwater rice	NO	-
807	Bangladesh	Synechococcus	S	deepwater rice	NO	-
769	Bangladesh	Synechococcus	S	deepwater rice	NO	-
838	North America	Synechococcus	S	lake	NO	-
562	North America	Synechococcus	S	pond	HM	-
33	North America	Synechococcus	S	pond	NO	-
797	Nepal	Synechococcus	S	paddy rice	NO	-
585	Iraq	Tolypothrix	N	paddy rice	CA	-

Abbreviations

Taxa: N - non-Rivulariaceae, S - Synechococcus, Y - Rivulariaceae.

Env (environment): HM - heavy metal, CA - calcareous, No - non-calcareous,

MAR - marine.

Hair: - absent, + present.

2.21 Eukaryotic algae

Three eukaryotic algal strains were used to compare localization of PMEase activity on eukaryotic and cyanobacterial hairs. Batrachospermum and Draparnaldia sp. were collected from Middleton Quarry, Teesdale and Lemanea was collected from Stanhope ford, Teesdale. The algae were washed in assay medium (2.532) and PMEase activity was localized using naphthol AS-MX phosphate (2.732).

2.3 CHEMICALS

2.31 Media

Reagents used in the preparation of media were of AnalaR grade, obtained from British Drug Houses Ltd. (BDH), Poole, Dorset.

2.32 Substrates used for assaying phosphatase activity

Table 2.2 Substrates used for assaying phosphatase activity.

Reagent	Supplier
(ρ-nitrophenyl phosphate disodium) (ρNPP)	Sigma Chemical co., USA.
bis (ρ-nitrophenyl) phosphate sodium salt (bis-ρNPP)	Sigma Chemical co., USA.
bispyridinium 2-methoxy-4-(2'-nitrovinyl)-phenyl phosphate (MNP)	King's College, London

2.4 COMMON PROCEDURES

2.41 pH

All pH measurements with a liquid volume of more than 2 ml, were carried out using an Ingold combination electrode and EIL pH meter (model 7050). For volumes below 2 ml, a Cardy compact pH meter C1 (Horiba, Ltd., Japan) was used. All probes were calibrated with BDH standard buffer solutions before measurements were taken. The buffers were arranged so that one was higher

and one lower than the pH of the solution under investigation. All standard buffer solutions were used at room temperature.

2.42 Light

Light measurements were taken with a Macam Lightmeter (model Q101). All incident light was measured as photosynthetically active radiation (PAR), and readings were recorded as photon flux density (μmol photon m^{-2} s^{-1}). The absence of light for P_i uptake experiments was achieved by covering flasks with 2 layers of aluminium foil followed by 2 layers of black polythene.

2.43 Adsorption

2.431 Shimadzu spectrophotometer

All large volume i.e. more than 300 μl colorimetric analyses were carried out using a Shimadzu Digital Double-Beam Spectrophotometer (model UV-150-2). Glass cuvettes with a path length between 1-10 cm were used for all readings between the visible and infra-red range of the spectrum.

2.432 MCC Plate Reader

The MCC Plate Reader was used for a large percentage of colorimetric analysis on alkaline phosphatase activity (APA). Assays using ρNPP and bis-ρNPP as substrates, Absorbance Program 1 and Filter Code 1 (405 nm) were used. Assays using MNP, Absorbance Program 1 and Filter Code 5 (510 nm) were used.

2.44 Assay for alkaline phosphatase activity

2.441 Preparation of material for analysis of alkaline phosphatase assays

Algae in batch culture were removed at 4-d intervals to investigate APA and cellular P content. Algae were removed carefully from the sides of the flask with a glass stirring rod, coated with silicon tubing. This procedure reduced cellular damage and prevented the release of cellular phosphatases

into the medium. The algae were centrifuged in 50 ml MSE non-sealable polyethylene centrifuge tubes, in a SS-34 8 x 100 ml angle head rotor, using a Sorvall RC-5B refrigerated superspeed centrifuge at 8000 x g for 20 min. The supernatant was decanted and made up to the required volume with MilliQ water, a 25 ml aliquot was stored at -20°C for future P analysis (2.61). The remaining medium was filtered via a GF/C filter (Whatman) and regarded as the extracellular phosphatase fraction.

The algal pellet was washed twice and resuspended in assay medium (2.533), which was 1.4 times its normal concentration This allowed for the dilution effect in the assay. The volume required to resuspend the algal pellet would vary depending upon the concentration of algae required in the assay. The algae were homogenized by passing them through a graded series of sterile syringe needles (Gillette Surgical Ltd., U.K.). The algae were sonicated in a MSE Soniprep 150 at an amplitude of 26 µm for 2 min. During sonication the algal homogenate was cooled with an ice jacket. The algal suspension was examined under the light microscope for cellular damage, previous results showed minimal cellular damage. The extensive homogenization procedure was required for two reasons. The first was repeatability, as the coefficient of variation in readings were between 5-10%, whereas in lightly homogenized material they were between 10-20%. Secondly non-homogenized material could interfere with the beam of light in the MCC Plate Reader. An aliquot of algal homogenate was washed in MilliQ and removed for dry wt (2.57) and P analysis (2.61), the remaining homogenate was assayed for APA.

2.442 Assay procedure for alkaline phosphatase activity using the MCC Plate Reader

For cell-bound and extracellular APA a 30 µl algal sample was pipetted via a Titertek 8-channel pipettman (EFLAB, Finland) into a 2% Decon washed (2.51) 96 microwell plate (no. 96F, Inter Med, NUNC, Denmark). Each sample had 8

replicates. All the microwell plates had lids (no. 96L, Inter Med, NUNC, Denmark), which reduced contamination, prevented evaporation and acted as an insulator against heat-loss. 90 µl of standard buffer was pipetted into the microwells i.e. glycine-NaOH, pH 10.3 (50 mM final concentration). The plate was then incubated at 32°C for 30 min. Then 180 µl of 0.25 mM ρNPP or 0.5 mM bis-ρNPP (7.75 mg l^{-1} P final concentration) was pipetted into the microwells. A T = 0 min reading was taken and subsequent readings at 10 min intervals for 30 min. There was no termination of APA. Subsequently a time course was plotted from the readings, and a value for APA was taken from the linear part of the constructed graph. A calibration curve using ρ-nitrophenol (ρNP) at pH 10.3 was constructed between 0.002-0.2 µmol. Activity was expressed as µmol ρNP mg dry wt^{-1} h^{-1}.

Alkaline phosphatase assays using MNP were similar to ρNPP and bis-ρNPP except the buffer used was 60 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris), containing 0.3 mM $MgSO_4$, 3 µm $ZnSO_4$ and 0.03% Bovine Serum Albumin (BSA) (final concentrations). The buffer was at pH 9.5, which was the optimum pH for the colour formation of the 2-methoxy-4-(2'-nitrovinyl)-phenol ring system released via hydrolysis by PMEases. The final concentration of MNP was 0.15 mM (4.65 mg l^{-1} P). There was no termination of APA in this assay. A calibration curve was constructed using 2-methoxy-4-(2'-nitrovinyl)-phenol between 0.01-2 µmol at pH 9.5. Activity was expressed as µmol PO_4-P hydrolyzed mg dry wt^{-1} h^{-1}.

2.45 Effect of pH on phosphatase activity

All assays were carried out using the MCC plate reader. The algae were prepared for analysis as in 2.441. At each pH unit duplicate buffers were used to compensate for any inhibition of APA by the buffers used (Table 2.3). The buffers were 50 mM (final concentration), which was chosen as a suitable concentration for buffering physiological media (Dawson et al., 1986). The

effectiveness of each buffer was tested during the assay run, using the Cardy compact pH meter C1 (2.41). The pH of each buffer varied +/- 0.05 of a pH unit. PMEase activity was monitored using ρNPP. For assaying PMEases 20 µl of alga and 70 µl of buffer were used. This was incubated at 32°C for 30 min. After the incubation period 140 µl of ρNPP substrate (pre-incubated to 32°C) was added. The assay ran for 30 min and was terminated by the addition of 100 µl of 4.95 M NaOH, resulting in a final pH of c. 12.8 +/- 0.1. At each pH a T = 0 line was set up by the addition of NaOH before ρNPP, which compensated for any optical variation.

A different protocol was used to assay for PDEase activity, because the addition of 100 µl of 4.95 M NaOH resulted in bis-ρNPP hydrolysis. To screen for PDEase activity 30 µl of alga; 90 µl of buffer and 180 µl of substrate were used. The assay was terminated with 30 µl of 0.33 M NaOH, resulting in a final pH of 12.3. For each of the above procedures calibration curves were set up at the requisite pH values using ρNP between 0.0002-0.2 µM.

Table 2.3 Buffers used to investigate the effect of pH on phosphatase activity. Two buffers were used at each pH value (A+B).

pH	buffer	final conc. (mM)	set	buffering capacity	pKa at 20°C
3.0	DMG-NaOH	50	A	3.2-7.6	3.66 and 6.20
3.0	glycine-HCL	50	B	2.2-3.6	2.35 and 9.60
4.0	DMG-NaOH	50	A	3.2-7.6	3.66 and 6.20
4.0	succinic acid	50	B	3.8-6.0	4.18 and 5.60
5.0	DMG-NaOH	50	A	3.2-7.6	3.66 and 6.20
5.0	succinic acid-NaOH	50	B	3.8-6.0	4.18 and 5.60
6.0	DMG-NaOH	50	A	3.2-7.6	3.66 and 6.20
6.0	succinic acid-NaOH	50	B	3.8-6.0	4.18 and 5.60
7.0	DMG-NaOH	50	A	3.2-7.6	3.66 and 6.20
7.0	HEPES-NaOH	50	B	6.8-8.2	7.50
8.0	TES-NaOH	50	A	6.8-8.2	7.50
8.0	HEPES-NaOH	50	B	6.8-8.2	7.50
9.0	AMeP-NaOH	50	A	9.0-10.5	9.69
9.0	glycine-NaOH	50	B	8.6-10.6	2.35 and 9.60
10.0	AMeP-NaOH	50	A	9.0-10.5	9.69
10.0	glycine-NaOH	50	B	8.6-10.6	2.35 and 9.60
10.3	AMeP-NaOH	50	A	9.0-10.5	9.69
10.3	glycine-NaOH	50	B	8.6-10.6	2.35 and 9.60
11.0	CAPS-NaOH	50	A	9.8-11.1	10.40
11.0	Na_2CO_3-$NaHCO_3$	50	B	9.2-10.8	10.33

2.5 STANDARD CULTURING TECHNIQUES

2.51 Cleaning of apparatus

All glassware and plastic apparatus were cleaned by soaking in 2% Decon 90, a phosphate free detergent, (Decon Laboratories Ltd.,England) for 20 min. The apparatus were rinsed six times in distilled water. All volumetric glassware was dried at room temperature, plastics were dried at 40°C and glassware at 100°C. Any apparatus which were difficult to clean were placed in hot Decon c. 80°C for 1 h and scrubbed clean.

2.52 Sterilization

Culture media and apparatus were sterilized by autoclaving at 121°C (10.35 KN m^{-2}; 15 psi) for 15 min. Solutions destabilized by heat were filter-sterilized through pre-sterilized 0.2 µm Millipore cellulose acetate filters. Isolation needles were sterilized by dipping in ethanol and flaming briefly.

2.53 Media

2.531 Chu 10D

The medium for the culturing of all cyanobacteria used was a modification of the number 10 of Chu (1942) as given by Gerloff et al. (1950). This medium is referred to as Chu 10D. To induce heterocyst formation the medium was made nitrogen free by the substitution of 35.83 mg l^{-1} $CaCl_2.2H_2O$ for $Ca(NO_3)_2.4H_2O$ this medium was referred to as Chu 10D-N. For the growth of all strains 1 mg l^{-1} $P\text{-}PO_4$ was used as a low phosphate medium and 10 mg l^{-1} $P\text{-}PO_4$ as high phosphate medium. The chelator ethylene diamine tetra-acetic acid (EDTA) was added to increase the availability of Fe^{3+} and decrease the precipitation of iron-phosphate salts. Media compositions are in Tables 2.4 and 2.5.

2.532 Assay medium

This was a further modification of Chu 10D-N medium, where N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) was removed, KH_2PO_4 was replaced with KCL and the iron-EDTA concentration was halved.

Table 2.4 Concentration of mineral salts in Chu 10D (Harding and Whitton, 1976) and assay medium.

Salt	Chu 10D-N		assay medium	
	mg l^{-1}	µM	mg l^{-1}	µM
$NaNO_3$	-	-	-	-
$Ca(NO_3)_2.4H_2O$	-	-	-	-
KH_2PO_4	4.3942	32.20	-	-
$MgSO_4.7H_2O$	25.0000	101.40	25.0000	101.40
$Na_2SiO_3.5H_2O$	-	-	-	-
$NaHCO_3$	15.8500	188.60	15.8500	188.60
$FeCl_3.6H_2O$	2.4250	8.97	1.2125	4.45
$Na_2EDTA.3H_2O$	3.3371	8.97	1.6685	4.45
$MnCl_2.4H_2O$	0.4525	2.28	0.4525	2.28
H_3BO_3	0.7150	115.60	0.7150	11.56
$Na_2MoO_2.2H_2O$	0.0067	0.02	0.0067	0.02
$ZnSO_4.7H_2O$	0.0555	0.19	0.0555	0.19
$CuSO_4.5H_2O$	0.0197	0.07	0.0197	0.07
$CoSO_4.7H_2O$	0.0105	0.03	0.0105	0.03
$NiSO_4.7H_2O$	0.0380	0.03	0.0380	0.03
NaOH	c. 60	1500.00	-	-
HEPES	600.0000	2517.00	-	-
KCl	1.8718	25.10	4.2784	57.38
$CaCl_2.2H_2O$	35.8300	243.70	35.8300	243.70

Table 2.5 Elemental compositions of media used.

Element	Chu 10D-N		assay medium	
	mg l^{-1}	µM	mg l^{-1}	µM
Cl	18.8812	532.50	20.4300	562.80
N	-	-	-	-
Ca	9.7674	243.70	9.7674	243.70
Na	39.2341	167.00	4.5432	197.60
S	3.2607	101.70	3.2607	101.70
Mg	2.4645	101.40	2.4645	101.40
K	2.2431	57.38	2.2431	57.38
Si	-	-	-	-
P	1.0000	32.28	-	-
B	0.1249	11.56	0.1249	11.56
Fe	0.5010	8.97	0.2505	4.48
Mn	0.1255	2.28	0.1255	2.28
Zn	0.0126	0.19	0.0126	0.19
Cu	0.0050	0.07	0.0050	0.07
Co	0.0022	0.03	0.0022	0.03
Ni	0.0019	0.03	0.0019	0.03
Mo	0.0026	0.02	0.0026	0.02

2.54 Preparation of media for batch culture

To prepare 1 litre of medium, 400 ml of MilliQ water was buffered with 0.6g (0.25 mM) of HEPES. HEPES was chosen because of its reported lack of interference in biological systems (Smith and Foy, 1974). Upon addition of HEPES the pH dropped to c. 5.0. This was adjusted to ph 7.6 by the gradual addition of 1.0 M NaOH. The buffered solution was transferred to a 1-litre volumetric flask. The media mineral salts were pipetted in a specific order (Table 2.4) to the buffered solution. Upon addition of each salt, the flask

was shaken gently to mix the salts and prevent any reaction due to an unusually high local concentration of salts. After addition of the salts the final volume was adjusted to 1 litre with MilliQ water. Medium was made up as it was required, from stock solutions stored at 4°C. Aliquots of 50 ml of medium were dispensed into 100 ml conical flasks. The flasks were plugged with silicon bungs (type S28, SANKO Plastic co. Ltd., Japan) and autoclaved.

2.55 Subculturing

All subculturing was carried out using standard aseptic techniques in a Microflow Pathfinder laminar flow cabinet conforming to B.S. 5295 class 1. The cabinet was sprayed with ethanol (98%) 15 min before use to reduce the possibility of contamination by air-borne bacteria and fungi. For routine subculturing clumps of algae were transferred to fresh medium via a sterile pasteur pipette. For experimental work a standard homogenous inoculum was used, which had been passed through a graded series of sterile syringe needles (2.441). From this suspension aliquots were transferred aseptically to each flask by automatic pipettman to give 10 mg dry wt l^{-1} concentration. All inocula, unless otherwise stated, were grown for 7 d in Chu 10D-N 1 mg l^{-1} P-PO_4 at 32°C and 100 µmol photon m^{-2} s^{-1}.

2.56 Incubation conditions

2.561 Stock cultures

Stock cultures were maintained in thermostatically controlled growth rooms at 25°C, with continuous overhead illumination (60 µmol photon m^{-2} s^{-1} PAR supplied by Phillips warm white fluorescent tubes). Stocks were also maintained at 10°C under continuous illumination (15 µmol m^{-2} s^{-1} PAR). Subculturing was carried out every 6 months.

2.562 Batch cultures

All of the experimental cultures, unless stated otherwise, were incubated in thermostatically controlled Gallenkamp shaker tanks, illuminated continuously from below by Phillips warm white fluorescent tubes. A shaking mechanism moved the flasks through a horizontal distance of 40 mm, 66 times min^{-1}. Incubation temperature was 32°C, unless otherwise stated. Flasks were suspended by the neck until approximately 1 cm of the base was submerged, giving a light intensity between 80-100 μmol photon m^{-2} s^{-1} depending upon the position of the flasks in the tank relative to the fluorescent tubes below. In order that each flask received an equal amount of light, positions were randomized every 2 d.

2.563 Phosphatase assays

Assays performed in universal bottles were incubated in the Gallenkamp growth tanks at 32°C or 25°C. Assays using microwell plates were incubated in a thermostatically controlled incubator.

2.57 Analysis of yield by dry weight and observation

For routine morphological studies algal material was collected via sterile pipette tips. To determine the dry wt and cellular P content an algal homogenate was prepared (2.441). A sample (>10 ml) was removed from the homogenate, centrifuged and washed twice with MilliQ water. The resultant algal pellet from the second washing was resuspended in 5 ml of MilliQ water. This suspension was placed in a 6 ml porcelain crucible (BDH). The crucible was previously 2% decon washed, dried at 105°C for 1 h, cooled in a desiccator and its weight determined to 5 decimal places on an Oertling balance (model R51). The algal suspension was dried at 105°C for 24 h and cooled in a desiccator for 1 h before determining the dry wt. The dried alga was then used for the determination of cellular P (2.61).

Observation of yield was scored between 0 - 5 every 4 d. Minimum growth, i.e. in -P, was scored 0 and maximum growth in P-PO_4 was scored 5 (Table 4.2). Death in batch culture was represented as -. Brief light microscope analysis on morphology was also carried out.

2.6 CHEMICAL ANALYSIS

2.61 Total filtrable phosphorus

Algal and media samples were determined for total filtrable phosphorus according to the method of Eisenreich et al. (1975). Samples were digested in 125 ml Erlenmeyer flasks, capped with aluminium foil and autoclaved at 10^5 Pascal for 30 min. Mean %recovery for phosphorus using this method was 94.2%.

2.62 Total carbohydrate content

The method of Dubois et al. (1956) was used to determine the carbohydrate content of extracellular fractions used in the purification of an extracellular phosphatase from Calothrix 550 (5.3). A 2 ml aqueous solution for carbohydrate determination, containing c. 10-80 µg sugar + 50 µl phenol solution (80 g phenol + 20 ml MilliQ water), was carefully added to 5 ml concentrated H_2SO_4. This whole procedure was carried out in a fume cupboard at 4°C. The solution stood for 30 min at room temperature. The orange-yellow colour formed was stable for several hours and was read at 480-490 nm. A calibration curve was constructed using glucose in the range 0-100 µg. This method was chosen as it is sensitive, quantitative, reproducible and there was no interference from protein.

2.7 MICROSCOPY

2.71 Light microscopy

In the laboratory material was examined using a type 109 Nikon Fluophot microscope, fitted with a Nikon micrometer eyepiece. Light micrographs were taken using a Nikon M-350 automatic exposure camera. Kodak Technical Pan film was used for black and white pictures and Kodak Ektachrome Tungsten Professional film was used for colour pictures.

To avoid osmotic effects live samples were mounted in the media they originated from. Fixed material was mounted in distilled water. Cell counts were made as random as possible by indiscriminate moving of the slide under investigation.

2.72 Morphology

2.721 Definition of a hair

Hairs were identified using the definition of Sinclair and Whitton (1977) as "a region of the trichome where the cells are narrow, elongated, highly vacuolated and usually apparently colourless".

2.722 Morphological scoring

The %hairiness of a sample was defined as the percentage of trichomes which possessed hairs, and was determined from a count of 100 trichomes selected at random.

2.73 Localization of phosphatase activity

2.731 Localization of phosphomonoesterase activity using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

Localization of PMEase activity was carried out using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as an organic P substrate (Coston and Holt, 1958; Holt and Withers, 1958). Material was washed three times and resuspended in

1 mM BCIP in assay medium (2.533) at pH 10.3 (50 mM glycine-NaOH) in a glass vial. The material was shaken frequently for 15 min at 32°C and then washed three times and examined under the microscope. This assay was usually carried out in a shaken glass vial as oxygen is required for formation of the insoluble blue indigoid.

2.732 Localization of phosphomonoesterase activity using naphthol AS-MX phosphate

Localization of PMEase activity was tested by microscopy using 3-hydroxy-2-naphthoic acid 2,4-dimethlyanilide phosphate sodium salt (naphthol AS-MX phosphate) as the organic P source and diazotized 4-benzoylamino-2,5-dimethoxyanilide zinc chloride salt (Fast Blue RR diazonium salt) as the coupling agent; the product is a violet insoluble dye. The staining medium consisted of 12.0 ml of assay medium (2.533), 0.5 ml naphthol AS-MX phosphate alkaline solution (SIGMA technical bulletin 85) and 7.5 mg of Fast Blue RR diazonium salt; a magnetic stirrer was used during the preparation. The staining medium was adjusted to pH 9.0 using 1 M NaOH and then filtered through a GF/C glass microfibre filter (Whatman); it was used immediately after preparation. Material was washed three times with assay medium, resuspended in the staining medium, left for 15 min at 32°C, washed three times in assay medium and examined under the microscope.

2.733 Localization of phosphodiesterase and 5′-nucleotide phosphodiesterase activity using β-naphthyl phenylphosphonate

Localization of PDEase and 5′-NDEase activity was achieved using β-naphthyl phenylphosphonate as the organic P source (Kelly et al., 1975) and diazotized 2-methyl-4-[(2-methylphenyl)azo]benzenediazonium sulphate salt (Fast Garnet GBC sulphate salt) as the coupling agent; the product is an orange insoluble dye. The staining medium consisted of assay medium at pH 9.0 (5 mM AMeP-

NaOH), 1 mM naphthyl phenylphosphonate and 0.1% Fast Garnet GBC sulphate salt. The staining medium was filtered through a 0.22 μm nitro-cellulose filter (Millipore) and used immediately after preparation. Material was washed three times in assay medium and resuspended in the staining medium, left for 15 min at 32°C, washed three times in assay medium and examined under the microscope. Truetouch gloves (Surgikos) were used throughout as Fast Garnet GBC sulphate salt is a possible carcinogen.

CHAPTER 3

UTILIZATION OF ORGANIC PHOSPHORUS SOURCES AND PHOSPHATASE ACTIVITIES IN 51 AXENIC CYANOBACTERIA

3.1 INTRODUCTION

Inducible phosphatase activity has been detected in a range of cyanobacterial strains (1.6). However, there has been no comparison of organic P utilization and phosphatase activities in different taxa and environments. Therefore a survey was carried on 51 cyanobacterial strains, which had been isolated from known physical and chemical environments, with the main emphasis on the Rivulariaceae. The survey comprised 30 Rivulariaceae, 14 filamentous non-Rivulariaceae and seven Synechococcus strains.

In order to distinguish between strains they were categorized into large taxon, genus, physical environment and chemical environment. As the role of hair cells was the main aim of the overall research, a further sub-category of hair-forming Rivulariaceae strains was included.

3.2 Method

The 51 cyanobacterial strains used are described in Table 2.1, which includes for each strain a list of the large taxon, genus, location, physical environment, chemical environment and the presence or absence of a hair. All cultures were grown in 10 ml of Chu 10D-N (P 1 mg l^{-1}) in 60-ml capacity boiling tubes slanted at 45° to increase gaseous exchange, capped with Axa closures (Axa Ltd.) and incubated at 32°C and 100 µmol photon m^{-2} s^{-1}. It was possible to grow all strains under these standard incubation conditions. However, it was taken into consideration, when analyzing the data, that this could lead to increased yields in some strains that were closer to their optimum growing conditions.

Material was subcultured at two four-d intervals, centrifuged (8000 x g), washed twice, resuspended in Chu 10D-N,-P (2.531) and inoculated at ca 10 mg l^{-1} dry wt. Four replicates were used for each experiment.

All organic P sources were filter sterilized through 0.22 µm Nuclepore filters and added at 1 mg l^{-1} P (Table 3.1). P sources were stored at -20°C. Cultures were grown to moderate P deficiency (16 d), harvested (2.441, 2.57) and the cultures grown in P_i were assayed at pH 7.6 (HEPES - NaOH buffer) and pH 10.3 (glycine - NaOH buffer) for cell-bound and extracellular PMEase and PDEase activities (2.45). Assays were run for 30 min or until yellow colouration of ρNP was observed; phosphatase substrates used were ρNPP (0.25 mM final concentration) and bis-ρNPP (0.5 mM final concentration). Localization of cell-bound PMEase and PDEase activity was carried out (when detectable) using naphthol AS-MX phosphate (2.732) and β-naphthyl phenylphosphonate (2.733), respectively. Activity was expressed as µmol ρNP mg dry wt^{-1} h^{-1}.

The yields in different P sources and phosphatase activities for each strain were placed in sequence and organized into groups (Fig. 3.1) based on taxonomic and environmental criteria. The means of the ranks of the groups were then compared. When the sample number was above five the Mann-Whitney U-test (Elliott, 1977) was used to compare two populations. The power efficiency of this test is never less than 86% (Elliott, 1977). A null hypothesis was drawn up that the two independent random samples were drawn from populations having the same parent distributions and the same medians. The significance of this hypothesis was rejected at the $P = <0.05$ level. When there were less than two significant values between the different categories tested the values are not tabulated. The Kruskal-Wallis one-way analysis by ranks was also used to compare more than two populations. The power efficiency of this test is ca. 96% (Elliott, 1977).

Table 3.1 Organic phosphorus sources used to screen 51 cyanobacterial strains

organic P source
sodium-β-glycerophosphate (β-gly)
ρ-nitrophenyl phosphate (ρNPP)
2-methoxy-4-(2′-nitrovinyl)-phenyl phosphate (MNP)
ATP
bis (ρ-nitrophenyl) phosphate (bis-ρNPP)
DNA
phytic acid (phy)

Fig. 3.1 Representation of the categories selected for the survey of yields and phosphatase activities in 51 cyanobacterial strains.

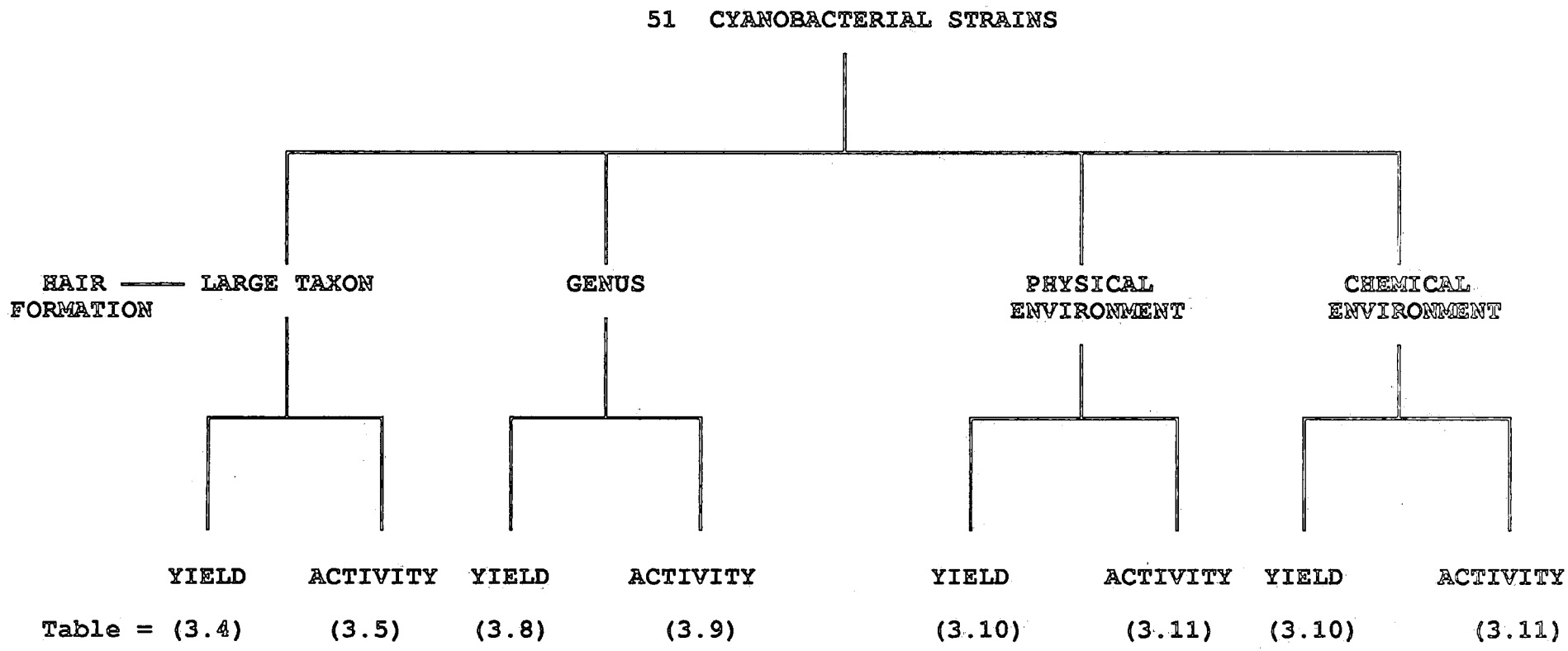


Table 3.2 Yields in 8 different phosphorus sources for 51 cyanobacterial strains. Pos = position relative to the 51 strains, no. = Durham Culture Collection number, loc = origin (country) of strain, gen = genus, tax = large taxon, phy = physical environment, che =chemical environment, ALB = Albania, ALD = Aldabra, BAN = Bangladesh, CUB = Cuba, FRA = France, FRG = West Germany, IND = India, IRQ = Iraq, NEP = Nepal, NZ = New Zealand, PHI = Philippines, SAU = Saudi Arabia, SL = Sri Lanka, TAI = Taiwan, THA = Thailand, UK = United Kingdom, USA = United States of America, ANA = Anabaena, CAL = Calothrix, CYL = Cylindrospermum, DIC = Dichothrix, FIS = Fischerella, GLO = Gloeotrichia, LYN = Lyngbya, NOS = Nostoc, SYN = Synechococcus, TOL = Tolypothrix, N = filamentous non-Rivulariaceae, R = Rivulariaceae, S = Synechococcus, CA = calcareous, HM = heavy metal, MAR = marine, NO = non-calcareous (others) DWR = deepwater rice, LAK = lake, PAD = paddy rice, PON = pond, SOI = soil, STR = stream, Y = yield and SD = standard deviation. Underlined strains form hairs when phosphorus deficient.

pos	no.	loc	gen	tax	phy	che	-P		PO_4		β-gly		ρNPP		MNP		DNA		bis		ATP		phy	
							Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD
1	743	THA	GLO	Y	DWR	NO	27.05		572.70		515.35		489.10		431.21		552.31		350.34		506.40		142.13	
2	750	THA	ANA	N	DWR	NO	107.13		562.49		398.37		400.41		232.22		386.36		383.41		334.38		300.32	
3	627	BAN	LYN	N	DWR	NO	108.19		536.49		376.32		390.37		365.44		350.28		366.35		452.41		163.17	
4	608	BAN	NOS	N	DWR	NO	104.17		518.48		376.34		259.27		256.19		370.34		289.17		399.47		313.29	
5	251	USA	CAL	Y	STR	NO	109.10		498.34		432.42		404.33		383.36		263.36		230.23		259.31		276.17	
6	764	BAN	CAL	Y	DWR	NO	78.06		493.33		293.29		345.31		197.30		88.04		300.50		60.09		95.05	
7	734	THA	NOS	N	DWR	NO	71.09		480.41		417.39		289.28		224.24		293.17		254.29		156.11		245.29	
8	614	BAN	NOS	N	DWR	NO	76.07		472.44		379.34		346.37		339.29		372.32		424.47		423.38		226.28	
9	800	UK	NOS	N	PON	NO	148.16		465.53		366.38		344.37		339.09		309.14		262.27		304.65		129.24	
10	184	UK	CAL	Y	STR	CA	104.20		452.17		478.13		327.21		390.23		489.25		377.27		20.03		201.12	
11	795	NEP	CAL	Y	PAD	NO	79.15		445.44		456.48		238.30		270.39		401.21		260.35		426.46		154.25	
12	253	CUB	CAL	Y	MAR	MAR	74.03		431.21		209.15		216.26		170.09		143.04		131.02		139.07		193.13	
13	794	NEP	CAL	Y	PAD	NO	69.13		431.09		406.21		385.35		365.41		404.33		323.05		411.14		258.34	
14	624	BAN	CAL	Y	DWR	NO	102.15		431.02		309.24		390.12		266.33		419.16		338.31		293.21		185.31	
15	611	BAN	NOS	N	DWR	NO	101.17		416.39		367.38		336.27		302.34		119.12		282.18		173.19		291.24	
16	33	USA	SYN	S	PON	NO	82.09		382.31		363.49		367.29		346.37		380.21		321.36		335.42		98.11	
17	254	SL	CAL	Y	PON	NO	88.28		378.11		376.63		368.60		372.64		395.61		213.41		382.42		162.27	
18	281	USA	GLO	Y	PON	NO	76.18		366.41		300.55		362.30		294.41		341.45		204.46		277.22		85.20	
19	602	BAN	GLO	Y	DWR	NO	49.07		361.15		351.23		268.34		219.14		219.19		259.17		169.35		38.09	
20	796	NEP	CAL	Y	PAD	NO	75.15		351.32		311.14		298.31		280.16		429.54		234.21		205.17		193.23	
21	730	THA	CAL	Y	DWR	NO	31.06		350.42		324.32		315.16		351.39		286.27		319.40		343.39		143.17	
22	182	IND	CAL	Y	PAD	NO	82.15		339.11		334.31		343.67		304.67		305.54		279.14		320.23		209.39	
23	808	ALB	DIC	Y	STR	CA	100.08		335.03		311.26		334.07		288.17		347.16		216.17		300.28		127.13	
24	626	BAN	GLO	Y	DWR	NO	58.07		330.23		298.23		325.26		312.15		287.14		262.27		262.18		161.10	

pos	no.	loc	gen	tax	phy	che	-P		PO_4		β-gly		ρNPP		MNP		DNA		bis		ATP		phy	
							Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD	Y	SD
25	809	NZ	DIC	Y	STR	NO	88.10		318.23		258.27		288.34		263.27		187.29		274.21		271.29		205.20	
26	746	THA	ANA	N	DWR	NO	28.03		318.22		115.13		68.07		63.11		60.06		78.09		126.15		105.07	
27	613	BAN	GLO	Y	DWR	NO	10.02		314.23		234.32		224.35		195.29		254.09		169.33		26.02		19.03	
28	255	USA	CAL	Y	PON	NO	130.29		310.23		319.13		293.13		313.28		326.30		242.21		325.31		291.37	
29	603	BAN	CAL	Y	DWR	NO	41.06		308.17		281.21		234.17		194.15		241.25		228.17		228.19		108.09	
30	202	ALD	CAL	Y	PON	CA	47.06		304.28		317.18		216.17		228.21		241.16		108.06		228.11		33.02	
31	612	BAN	FIS	N	DWR	NO	70.12		301.29		281.17		290.32		288.17		249.19		218.22		303.27		98.08	
32	266	FRA	CAL	Y	STR	CA	81.04		300.31		258.17		243.06		226.22		288.17		168.09		299.16		122.08	
33	786	IND	CAL	Y	SOI	NO	64.07		293.24		248.17		265.12		263.22		189.11		253.17		15.01		110.13	
34	217	ALD	ANA	N	PON	CA	70.09		287.29		201.11		238.16		156.17		191.12		182.13		265.22		131.11	
35	802	BAN	CAL	Y	DWR	NO	90.11		281.36		270.22		258.29		252.13		290.13		176.04		248.23		165.09	
36	585	IRQ	TOL	N	PAD	CA	58.08		277.22		205.15		277.12		267.16		264.27		266.21		257.29		163.15	
37	690	SAU	CAL	Y	STR	CA	13.02		272.26		248.28		257.31		245.19		291.35		231.27		51.05		244.22	
38	572	PHI	GLO	Y	PAD	NO	30.02		268.13		263.40		250.31		267.47		250.09		189.12		220.10		167.10	
39	550	UK	CAL	Y	STR	CA	52.05		262.21		291.24		233.13		203.18		250.21		216.24		15.02		167.08	
40	694	TAI	CAL	Y	PAD	NO	24.04		254.24		170.15		207.19		152.10		188.14		161.15		150.30		23.05	
41	201	ALD	NOS	N	PON	CA	64.08		250.17		220.21		129.13		216.24		159.16		199.22		181.11		97.07	
42	688	SAU	CAL	Y	STR	CA	40.07		248.23		231.13		174.31		176.15		186.17		151.14		35.05		33.04	
43	767	BAN	SYN	S	DWR	NO	15.00		227.00		69.00		166.00		179.00		39.00		72.00		204.00		34.00	
44	807	BAN	SYN	S	DWR	NO	17.00		200.00		160.00		81.00		200.00		41.00		27.00		189.00		10.00	
45	669	USA	ANA	N	PON	NO	41.04		181.07		158.12		130.07		139.11		143.14		130.17		142.08		172.07	
46	689	SAU	DIC	Y	STR	CA	13.02		153.08		189.22		174.09		180.13		103.11		153.07		63.07		101.09	
47	684	FRG	CYL	N	PON	NO	28.03		143.13		118.11		128.14		138.07		94.09		102.12		137.14		78.05	
48	838	USA	SYN	S	LAK	NO	15.00		128.00		107.00		83.00		151.00		96.00		37.00		29.00		10.00	
49	769	BAN	SYN	S	DWR	NO	15.00		114.00		107.00		87.00		47.00		68.00		74.00		108.00		10.00	
50	797	NEP	SYN	S	PAD	NO	18.00		104.00		128.00		150.00		58.00		56.00		83.00		10.00		10.00	
51	562	USA	SYN	S	PON	HM	9.00		78.00		57.00		83.00		62.00		77.00		15.00		13.00		10.00	

Table 3.3 Cell-bound and extracellular phosphomonoesterase and phosphodiesterase activity at pH 7.6 and pH 10.3 for 51 cyanobacterial strains. Pos = position relative to the 51 strains, no. = Durham Culture Collection number, loc = country of strain, gen = genus, tax = large taxon, phy = physical environment, che = chemical environment, ALB = Albania, ALD = Aldabra, BAN = Bangladesh, CUB = Cuba, FRA = France, FRG = West Germany, IND = India, IRQ = Iraq, NEP = Nepal, NZ = New Zealand, PHI = Philippines SAU = Saudi Arabia, SL = Sri Lanka, TAI = Taiwan, THA = Thailand, UK = United Kingdom, USA = United States of America, ANA = Anabaena, CAL = Calothrix, CYL = Cylindrospermum, DIC = Dichothrix, FIS = Fischerella, GLO = Gloeotrichia, LYN = Lyngbya, NOS = Nostoc, SYN = Synechococcus, TOL = Tolypothrix, N = filamentous non-Rivulariaceae, R = Rivulariaceae, S = Synechococcus, CA = calcareous, HM = heavy metal, MAR = marine, NO = non-calcareous (others) DWR = deepwater rice, LAK = lake, PAD = paddy rice, PON = pond, SOI = soil, STR = stream, PME 10.3 = cell-bound phosphomonoesterase activity at pH 10.3, PME 7.6 = cell-bound phosphomonoesterase at pH 7.6, XC = extracellular, PDE 10.3 = cell-bound phosphodiesterase activity at pH 10.3 and PDE 7.6 = cell-bound phosphodiesterase activity at pH 7.6., A = phosphatase activity, SD = standard deviation. Underlined strains form hairs whe phosphorus deficient.

pos	no.	loc	gen	tax	phy	che	PME 10.3		PME 7.6		XCPME 10.3		XCPME 7.6		PDE 10.3		PDE 7.6	
							A	SD	A	SD	A	SD	A	SD	A	SD	A	SD
1	602	BAN	GLO	Y	DWR	NO	15.73	,0.412	8.17	,0.264	0.14	,0.012	0.001	,0.001	2.56	,0.029	1.16	,0.026
2	585	IRQ	TOL	N	PAD	CA	15.58	,0.145	1.70	,0.130	0.55	,0.011	0.021	,0.001	2.12	,0.161	0.62	,0.065
3	611	BAN	NOS	N	DWR	NO	13.54	,1.107	6.31	,0.242	3.50	,0.265	1.70	,0.054	2.08	,0.085	0.54	,0.043
4	281	USA	GLO	Y	PON	NO	12.35	,0.064	0.68	,0.107	19.94	,0.152	2.19	,0.079	0.001	,0.001	0.001	,0.001
5	802	BAN	CAL	Y	DWR	NO	12.19	,1.001	7.28	,1.096	2.52	,0.326	0.48	,0.017	1.96	,0.048	0.56	,0.021
6	613	BAN	GLO	Y	DWR	NO	12.09	,0.549	5.50	,0.583	0.17	,0.027	0.001	,0.001	3.20	,0.043	1.20	,0.042
7	794	NEP	CAL	Y	PAD	NO	10.83	,0.215	1.44	,0.020	1.44	,0.021	0.15	,0.013	0.54	,0.026	0.92	,0.037
8	184	UK	CAL	Y	STR	CA	10.65	,0.550	3.16	,0.433	0.75	,0.041	0.30	,0.007	0.08	,0.002	0.18	,0.004
9	182	IND	CAL	Y	PAD	NO	9.45	,0.743	3.22	,0.018	0.94	,0.077	0.07	,0.001	1.94	,0.142	0.001	,0.001
10	550	UK	CAL	Y	STR	CA	8.95	,0.425	0.98	,0.111	1.09	,0.118	0.61	,0.016	1:02	,0.045	1.48	,0.046
11	255	USA	CAL	Y	PON	NO	8.86	,0.092	0.98	,0.072	15.05	,1.033	1.94	,0.012	0.001	,0.001	0.001	,0.001
12	562	USA	SYN	S	PON	HM	8.54	,0.086	0.66	,0.020	28.35	,0.222	1.83	,0.083	0.26	,0.024	0.001	,0.001
13	202	ALD	CAL	Y	PON	CA	8.15	,0.983	2.24	,0.312	0.001	,0.001	0.001	,0.001	0.96	,0.023	0.001	,0.001
14	690	SAU	CAL	Y	STR	CA	7.68	,0.218	0.28	,0.002	0.76	,0.087	0.001	,0.001	0.001	,0.001	0.074	,0.002
15	808	ALB	DIC	Y	STR	CA	7.18	,0.291	0.94	,0.016	0.27	,0.018	0.06	,0.004	0.28	,0.012	0.36	,0.004
16	626	BAN	GLO	Y	DWR	NO	6.83	,0.360	5.55	,0.015	0.52	,0.033	0.45	,0.041	1.92	,0.124	0.40	,0.022
17	266	FRA	CAL	Y	STR	CA	6.65	,0.327	0.90	,0.012	0.11	,0.003	0.001	,0.001	0.14	,0.008	0.16	,0.002
18	251	USA	CAL	Y	STR	NO	6.19	,0.363	4.72	,0.133	0.50	,0.025	0.22	,0.023	0.10	,0.016	0.092	,0.012
19	254	SL	CAL	Y	PON	NO	5.70	,0.096	1.21	,0.133	0.65	,0.045	0.14	,0.002	0.16	,0.024	0.001	,0.001

pos	no.	loc	gen	tax	phy	che	PME 10.3		PME 7.6		XCPME 10.3		XCPME 7.6		PDE 10.3		PDE 7.6	
							A	SD	A	SD	A	SD	A	SD	A	SD	A	SD
20	253	CUB	CAL	Y	MAR	MAR	5.25	,0.485	0.001	,0.005	0.001	,0.001	0.001	,0.001	0.82	,0.021	0.001	,0.001
21	795	NEP	CAL	Y	PAD	NO	5.12	,0.300	1.40	,0.130	0.19	,0.022	0.001	,0.001	0.001	,0.001	0.001	,0.001
22	807	BAN	SYN	S	DWR	NO	4.33	,0.172	0.58	,0.015	0.31	,0.013	0.089	,0.004	0.001	,0.001	0.262	,0.002
23	688	SAU	CAL	Y	STR	CA	4.32	,0.110	0.15	,0.011	2.91	,0.077	0.37	,0.024	0.001	,0.001	0.001	,0.001
24	734	THA	NOS	N	DWR	NO	4.29	,0.146	2.04	,0.172	0.91	,0.017	0.63	,0.012	0.26	,0.022	0.06	,0.001
25	572	PHI	GLO	Y	PAD	NO	4.25	,0.061	0.19	,0.004	1.14	,0.063	0.001	,0.001	0.001	,0.001	0.074	,0.003
26	217	ALD	ANA	N	PON	CA	4.11	,0.299	0.77	,0.017	0.89	,0.022	0.018	,0.001	0.66	,0.037	0.42	,0.034
27	603	BAN	CAL	Y	DWR	NO	4.09	,0.080	6.94	,0.240	1.25	,0.018	1.98	,0.082	3.02	,0.202	1.08	,0.082
28	764	BAN	CAL	Y	DWR	NO	4.00	,0.502	2.29	,0.216	0.50	,0.012	0.001	,0.001	4.50	,0.504	2.02	,0.280
29	624	BAN	CAL	Y	DWR	NO	3.90	,0.031	2.91	,0.012	1.58	,0.079	1.70	,0.087	1.90	,0.042	0.64	,0.023
30	730	THA	CAL	Y	DWR	NO	3.33	,0.201	5.11	,0.084	0.21	,0.026	0.15	,0.034	1.78	,0.028	1.36	,0.066
31	689	SAU	DIC	Y	STR	CA	2.73	,0.300	0.001	,0.001	0.98	,0.001	0.001	,0.001	0.001	,0.001	0.076	,0.003
32	743	THA	GLO	Y	DWR	NO	2.37	,0.067	2.44	,0.012	0.66	,0.021	1.28	,0.041	1.84	,0.084	1.14	,0.024
33	796	NEP	CAL	Y	PAD	NO	2.12	,0.057	1.88	,0.028	0.12	,0.011	0.15	,0.037	0.001	,0.001	0.001	,0.001
34	201	ALD	NOS	N	PON	CA	1.99	,0.019	0.73	,0.067	3.48	,0.092	0.42	,0.012	0.001	,0.001	0.18	,0.002
35	612	BAN	FIS	N	DWR	NO	1.29	,0.010	0.001	,0.001	0.11	,0.013	0.001	,0.001	0.18	,0.023	0.34	,0.020
36	669	USA	ANA	N	PON	NO	1.29	,0.011	0.46	,0.090	3.32	,0.292	0.56	,0.004	0.001	,0.001	0.001	,0.001
37	684	FRG	CYL	N	PON	NO	1.29	,0.010	1.77	,0.087	0.39	,0.017	0.001	,0.001	0.001	,0.001	0.001	,0.001
38	769	BAN	SYN	S	DWR	NO	1.26	,0.010	0.77	,0.013	0.20	,0.004	0.24	,0.002	0.368	,0.038	0.001	,0.001
39	694	TAI	CAL	Y	PAD	NO	1.25	,0.047	1.84	,0.155	0.42	,0.034	0.53	,0.035	0.52	,0.042	0.20	,0.040
40	614	BAN	NOS	N	DWR	NO	1.11	,0.057	1.10	,0.011	0.67	,0.058	0.75	,0.017	0.22	,0.021	0.14	,0.002
41	786	IND	CAL	Y	SOI	NO	1.11	,0.039	0.001	,0.001	0.001	,0.001	0.001	,0.001	0.001	,0.001	0.001	,0.001
42	809	NZ	DIC	Y	STR	NO	1.10	,0.090	0.11	,0.022	0.09	,0.001	0.001	,0.001	0.22	,0.002	0.001	,0.001
43	608	BAN	NOS	N	DWR	NO	1.06	,0.011	1.20	,0.049	0.87	,0.082	3.02	,0.313	0.40	,0.023	0.28	,0.032
44	797	NEP	SYN	S	PAD	NO	1.03	,0.034	0.29	,0.010	0.93	,0.004	0.24	,0.004	0.22	,0.016	0.148	,0.016
45	33	USA	SYN	S	PON	NO	0.46	,0.021	0.31	,0.019	1.91	,0.071	0.17	,0.018	0.138	,0.003	0.001	,0.001
46	750	THA	ANA	N	DWR	NO	0.26	,0.010	0.26	,0.005	0.074	,0.007	0.202	,0.013	0.001	,0.001	0.038	,0.003
47	838	USA	SYN	S	LAK	NO	0.22	,0.034	0.38	,0.013	0.051	,0.002	0.041	,0.001	0.001	,0.001	0.404	,0.008
48	627	BAN	LYN	N	DWR	NO	0.084	,0.001	0.23	,0.016	0.15	,0.015	0.32	,0.061	0.001	,0.001	0.001	,0.001
49	746	THA	ANA	N	DWR	NO	0.081	,0.001	0.001	,0.001	0.04	,0.005	0.001	,0.001	0.001	,0.001	0.001	,0.001
50	767	BAN	SYN	S	DWR	NO	0.073	,0.001	0.31	,0.024	0.45	,0.007	0.46	,0.002	0.204	,0.028	0.001	,0.001
51	800	UK	NOS	N	PON	NO	0.041	,0.002	0.29	,0.005	0.024	,0.001	0.18	,0.008	0.10	,0.007	0.21	,0.015

3.3 Results

The mean yields in the organic P sources and phosphatase activities for the 51 cyanobacterial strains are presented in Tables 3.2 and 3.3, respectively. The means of the yields were used instead of the original four samples to calculate the mean rank of the different categories selected, because the coefficient of variation amongst the four samples was low (<15%). Yields in the 51 strains ranged between 572 mg l^{-1} dry wt in Gloeotrichia 743 (P_i, Table 3.2) to 10 mg l^{-1} dry wt (no detectable growth) in five out of the seven Synechococcus strains cultured in phytic acid (Table 3.2). Cell-bound PMEase activity at pH 10.3 was detected in all strains and activity ranged from 15.73 to 0.041 μmol ρNP mg dry wt^{-1} h^{-1}. In all cases the level of cell-bound PMEase activity was higher than cell-bound PDEase activity (Table 3.3). Extracellular PMEase activity was detected in 48 strains at either pH 10.3 and/or pH 7.6, whereas extracellular PDease activity was only detected in six strains.

One pattern analyzed was the relationship between large taxon and yield (Table 3.4) and large taxon and phosphatase activities (Table 3.5). The taxonomic groupings were filamentous non-Rivulariaceae (N, n = 14), Rivulariaceae (Y, n = 30), Synechococcus (S, n = 7) and hair-forming Rivulariaceae (H, n = 9).

Table 3.4 Large taxon versus yield in organic phosphorus sources for 51 cyanobacterial strains.

	mean rank of large taxon			
P-source	N(n = 14)	Y(n = 30)	S(n = 7)	H(n = 9)
β-gly	24.6	22.7	42.8	21.3
ρNPP	25.3	22.8	41.0	24.3
MNP	27.0	22.3	39.7	21.8
ATP	21.5	25.6	36.2	30.4
bis-ρNPP	21.9	24.0	42.5	26.0
DNA	26.5	22.0	42.1	23.4
phy	19.9	24.1	46.1	16.0

Table 3.5 Large taxon versus cell-bound and extracellular phosphatase activities for 51 cyanobacterial strains. Cultures grown in inorganic phosphorus for 16 d (phosphorus deficient). XC = extracellular, CB = cell-bound.

	mean rank of large taxon			
phosphatases	N(n = 14)	Y(n = 30)	S(n = 7)	H(n = 9)
CB PMEase, pH 10.3	33.9	19.7	36.8	18.7
CB PMEase, pH 7.6	30.2	21.8	35.2	30.6
XC PMEase, pH 10.3	26.7	25.9	24.5	29.7
XC PMEase, pH 7.6	23.5	28.1	22.0	33.8
CB PDEase, pH 10.3	29.6	23.1	30.8	29.6
CB PDEase, pH 7.6	27.8	24.0	30.5	25.0

Table 3.6 Comparison of large taxon versus yield using the Mann-Whitney U-test.

	significance			
P-source	N vs Y	Y vs S	S vs N	H vs non-H
β-gly	N.S.	<0.01	N.S.	N.S.
ρNPP	N.S.	<0.01	N.S.	N.S.
MNP	N.S.	<0.01	N.S	N.S.
ATP	N.S.	N.S.	N.S.	<0.05
bis-ρNPP	N.S.	<0.01	<0.01	N.S.
DNA	N.S.	<0.01	<0.01	N.S.
phy	N.S.	<0.001	<0.001	<0.05

Table 3.7 Comparison of large taxon versus phosphatase activity using the Mann-Whitney U-test.

	significance			
phosphatases	N vs Y	Y vs S	S vs N	H vs non-H
CB PMEase, pH 10.3	<0.01	<0.01	N.S.	N.S.
CB PMEase, pH 7.6	N.S.	<0.05	N.S.	<0.05
XC PMEase, pH 10.3	N.S.	N.S.	N.S.	N.S.
XC PMEase, pH 7.6	N.S.	N.S.	N.S.	N.S.
CB PDEase, pH 10.3	N.S.	N.S.	N.S.	N.S.
CB PDEase, pH 7.6	N.S.	N.S.	N.S.	N.S.

Synechococcus was the least effective large taxon at utilizing 6 of the organic P sources, except for ATP, where no differences between large taxa were detected (Table 3.4 and 3.6). Comparisons of the mean ranks for yields in ATP (Table 3.4) suggest there is an improved ability for Synechococcus and non-Rivulariaceae strains to utilize ATP and a corresponding reduction in the efficiency of Rivulariaceae strains to utilize ATP. The inability of Synechococcus to hydrolyze the phosphodiesters DNA and bis-ρNPP and phytic acid was very significant ($P = <0.001$). No significant differences were shown in the utilization of the organic P sources between Rivulariaceae and non-Rivulariaceae (Table 3.4 and 3.6). Hair-forming Rivulariaceae were significantly more effective at utilizing phytic acid than non hair-forming Rivulariaceae ($P = <0.05$) and significantly less effective at utilizing ATP ($P = <0.05$) (Table 3.7).

From the data in Tables 3.2, 3.4 and 3.6 five out of the Synechococcus strains were unable to grow in phytic acid and Synechocoocus as a taxon was the least effective at hydrolyzing phytic acid. As the yields in the Synechococcus strains were low (Table 3.2) it was possible that the incubation conditions used were unsuitable for growth of these strains. Therefore, under more favourable incubation conditions the strains may then be able to grow using phytic acid as a P source. However, when the strains were grown under shaken, stirring and aerated conditions, to increase CO_2, with phytic acid there was no increase in yield.

The Rivulariaceae had significantly greater cell-bound PMEase activity at pH 10.3 than non-Rivulariaceae ($P = <0.01$, Table 3.7) and Synechococcus ($P = <0.01$, Table 3.7). However, at pH 7.6 the differences in cell-bound PMEase activity were not significant between the Rivulariaceae and non-Rivulariaceae, and the difference between Synechococcus and Rivulariaceae was reduced ($P = <0.05$, Table 3.7). There was no significant difference in cell-bound PMEase activity at pH 10.3 between hair-forming and non hair-forming Rivulariaceae (Table 3.7). However cell-bound PMEase activity at pH 7.6 significantly decreased in hair-forming strains ($P = <0.05$).

Rivulariaceae had the highest rank in terms of cell-bound PDEase activity (Table 3.5), although no significant differences in PDEase activities between large taxon were shown. Extracellular PDEase activity was only detected in Calothrix 603 and 624, Gloeotrichia 743 and in Nostoc 608, 611 and 614. Due to the absence of extracellular PDEase activity in the majority of strains it was not included as a criterion in the screen. Comparisons of yield and activity between the large taxon were not significant using the Kruskal-Wallis test, which suggests that there was a large overlap between the taxa.

Further trends were analyzed between yield and genus (Table 3.6) and phosphatase activity and genus (Table 3.7). Genera used were Anabaena (Ana, $n = 4$), Calothrix (Cal, $n = 21$), Cylindrospermum (Cyl, $n = 1$), Dichothrix (Dic, $n = 3$), Fischerella (Fis, $n = 1$), Gloeotrichia (Glo, $n = 6$), Lyngbya (Lyn, $n = 1$), Nostoc (Nos, $n = 6$), Synechococcus (Syn, $n = 7$), Tolypothrix (Tol, $n = 1$).

Table 3.8 Genus versus yield in organic phosphorus sources for 51 cyanobacterial strains.

	mean rank of genus									
P-source	Ana n=4	Cal n=21	Cyl n=1	Dic n=3	Fis n=1	Glo n=6	Lyn n=1	Nos n=6	Syn n=7	Tol n=1
β-gly	34.0	21.8	46.0	31.3	27.0	21.3	9.0	14.3	42.8	39.0
ρNPP	32.5	23.1	46.0	31.3	27.0	21.3	4.0	21.6	41.0	24.0
MNP	41.5	22.6	46.0	26.0	17.0	19.5	6.0	20.3	39.7	21.0
ATP	26.2	26.1	38.0	25.0	14.0	24.3	2.0	20.0	36.2	23.0
bis-ρNPP	31.0	23.8	44.0	27.0	27.0	23.3	4.0	15.5	49.6	15.0
DNA	32.0	21.2	44.0	30.3	29.0	20.5	12.0	22.3	42.1	24.0
phy	19.5	21.2	40.0	24.6	35.0	32.1	20.0	14.1	46.1	21.0

Table 3.9 Genus versus cell-bound and extracellular phosphomonoesterase and phosphodiesterase activities at pH 10.3 and 7.6 for 51 cyanobacterial strains.

	mean rank of genus									
phosphatase	Ana n=4	Cal n=21	Cyl n=1	Dic n=3	Fis n=1	Glo n=6	Lyn n=1	Nos n=6	Syn n=7	Tol n=1
CB PMEase, pH 10.3	39.0	20.0	36.0	29.3	37.0	14.0	48.0	32.6	36.8	2.0
CB PMEase, pH 7.6	39.0	20.7	18.0	40.3	49.0	16.6	43.0	22.8	35.2	19.0
XC PMEase, pH 10.3	29.2	25.8	32.0	31.0	43.0	24.0	39.0	19.6	24.5	26.0
XC PMEase, pH 7.6	30.0	27.3	41.0	39.3	46.0	25.0	19.0	11.3	22.0	35.0
CB PDEase, pH 10.3	36.2	23.4	48.0	31.6	29.0	18.0	40.0	24.5	30.8	6.0
CB PDEase, pH 7.6	34.7	25.6	48.0	27.6	17.0	16.8	46.0	21.6	35.6	10.0

No significant differences were detected between genus (Calothrix, Gloeotrichia and Nostoc) and yield and genus and activity using either the Mann-Whitney U-test and the Kruskal-Wallis one-way analysis, except for significantly higher extracellular PMEase activity at pH 7.6 in Nostoc compared to Calothrix ($P = <0.05$). Therefore when differences are discussed they are based only on a comparison of the mean rank values (Table 3.8 and 3.9. Nostoc had the highest yields in organic P sources and Synechococcus had the lowest yields (Table 3.8). Anabaena was effective at utilizing phytic acid, although Gloeotrichia was not. Calothrix was effective at utilizing all of the organic P sources, except for ATP (cf hairs, Table 3.5).

Highest cell-bound PMEase activities at pH 7.6 and pH 10.3 were in Gloeotrichia and Calothrix (Table 3.9). Nostoc had higher levels of cell-bound and extracellular PMEase activity at pH 7.6 compared with pH 10.3.

Yields in organic P sources and phosphatase activities were compared with the isolates original physical and chemical environment. Physical environments were categorized into deepwater rice, lake, marine, paddy rice, pond, soil and stream. Chemical environments were separated into calcareous, heavy metal sites, marine and non-calcareous (others).

Table 3.10 Physical (a) and chemical (b) environment versus yield in organic phosphorus sources for 51 cyanobacterial strains. cal = calcareous, DWR = deepwater rice, HM = heavy metal, mar = marine, non = non-calcareous (others).

(a) mean rank of physical environment

P-source	DWR n= 20	lake n= 1	marine n= 1	paddy n= 8	pond n= 11	soil n= 1	stream n= 9
β-gly	23.1	49.0	38.0	25.1	28.1	33.0	25.2
ρNPP	23.3	48.0	38.0	25.8	28.0	26.0	25.7
MNP	25.7	45.0	42.0	23.7	26.8	24.0	19.5
ATP	22.9	45.0	37.0	23.0	23.4	48.0	32.8
bis-ρNPP	21.5	49.0	41.0	24.0	32.1	21.0	26.5
DNA	26.4	43.0	40.0	20.8	26.5	34.0	24.6
phy	25.8	47.0	14.0	23.3	30.9	31.0	21.1

(b) mean rank of chemical environment

P-source	cal n = 11	HM n = 1	mar n = 1	non n = 38
β-gly	29.6	51.0	38.0	23.9
ρNPP	31.3	49.0	38.0	23.5
MNP	28.6	49.0	42.0	24.2
ATP	32.1	50.0	37.0	23.2
bis-ρNPP	29.4	51.0	41.0	23.9
DNA	26.0	46.0	40.0	25.0
phy	27.4	48.0	14.0	25.0

Table 3.11 Physical (a) and chemical (b) environment versus cell-bound and extracellular phosphatase activities for 51 cyanobacterial strains.

(a) mean rank of physical environment

phosphatases	DWR n= 20	lake n= 1	marine n= 1	paddy n= 8	pond n= 11	soil n= 1	stream n= 9
CB PMEase, pH 10.3	28.0	47.0	20.0	22.5	26.0	40.0	16.1
CB PMEase, pH 7.6	20.6	36.0	47.0	23.1	28.8	50.0	30.8
XC PMEase, pH 10.3	28.0	46.0	50.0	24.0	18.0	51.0	25.2
XC PMEase, pH 7.6	21.7	34.0	50.0	29.5	22.4	51.0	30.4
CB PDEase, pH 10.3	19.6	44.0	16.0	26.7	28.9	51.0	32.3
CB PDEase, pH 7.6	19.4	14.0	36.0	27.8	34.5	51.0	25.8

(b) mean rank of chemical environment

phosphatases	cal n = 11	HM n = 1	mar n = 1	non n = 38
CB PMEase, pH 10.3	17.5	12.0	20.0	28.9
CB PMEase, pH 7.6	28.8	33.0	47.0	24.4
XC PMEase, pH 10.3	23.0	1.0	50.0	26.8
XC PMEase, pH 7.6	31.0	5.0	50.0	24.4
CB PDEase, pH 10.3	28.0	23.0	16.0	25.7
CB PDEase, pH 7.6	22.5	38.0	36.0	26.4

Table 3.12 Comparison of physical environment versus phosphatase activity using the Mann-Whitney U-test

	significance					
phosphatases	DWR v PAD	PAD v PON	PON v STR	STR V PAD	STR V DWR	DWR V PON
CB PMEase, pH 10.3	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
CB PMEase, pH 7.6	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
XC PMEase, pH 10.3	N.S.	<0.05	<0.05	N.S.	N.S.	<0.01
XC PMEase, pH 7.6	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
CB PDEase, pH 10.3	N.S.	N.S.	N.S.	N.S.	<0.01	<0.05
CB PDEase, pH 7.6	N.S.	N.S.	N.S.	N.S.	N.S.	<0.01

No significant differences were shown between environment and yield or chemical environment and activity using the Mann-Whitney U-test or Kruskal-Wallis one way analysis. The highest ranks for yields in the phosphodiesters bis-pNPP and DNA were in deepwater and paddy rice isolates, although they were not highly significant (Table 3.10a). Stream isolates were the least

effective of the strains at utilizing ATP. This pattern in stream isolates is very similar to the trend in hair-forming Rivulariaceae (Table 3.4 and 3.7) as seven of the hair-forming Rivulariaceae strains originate from streams. Stream isolates had the highest rank of cell-bound PMEase activity at pH 10.3 (c.f. hair-forming strains Table 3.3) and reduced PMEase activities at pH 7.6 (Table 3.11a).

Deepwater rice isolates had significantly higher cell-bound PDEase activities than stream ($P = <0.01$) and pond ($P = <0.01$) isolates (Table 3.12). Pond isolates had significantly higher levels of extracellular PMEase activity at pH 10.3 than deepwater rice ($P = 0.01$), paddy rice ($P = 0.05$) and stream ($P = 0.05$) isolates (Table 3.12).

Non-calcareous strains were slightly more effective at hydrolyzing organic P sources than strains originating from calcareous environments (Table 3.10b). However, calcareous cell-bound PMEase (pH 10.3) and PDEase (pH 7.6) activities had higher ranks than non-calcareous activities (Table 3.11b).

Two broad patterns of PMEase localization were found amongst the strains able to hydrolyze naphthol AS-MX phosphate (Table 3.13). Seven of the nine hair-forming strains had PMEase activity localized only on the hairs. However, in Calothrix parietina 550 and 184 the reverse staining pattern occurred, where the basal end of the trichome stained and the hair cells did not. All other non hair-forming cyanobacterial strains had PMEase activity localized throughout the trichomes/cells with no distinct localization on certain areas or cells. PDEase activity, when detected, had no specific areas of localization in any of the cyanobacterial strains tested.

Table 3.13 Staining of PMEase and PDEase activity using naphthol AS-MX phosphate (PMEase) and β-naphthyl phenylphosphonate (PDEase) in 51 cyanobacterial strains. Assays at 32°C for 15 min.

No.	Genus	PMEase	PDEase	No.	Genus	PMEase	PDEase
750	Anabaena	-	-	684	Cylindrospermum	-	+
669	Anabaena	-	+	809	Dichothrix	+	+
217	Anabaena	-	+	808	Dichothrix	+	+
746	Anabaena	-	-	689	Dichothrix	-	-
255	Calothrix	+	-	612	Fischerella	-	+
251	Calothrix	+	+	572	Gloeotrichia	+	-
794	Calothrix	+	+	626	Gloeotrichia	+	+
182	Calothrix	+	+	743	Gloeotrichia	-	+
184	Calothrix	+	+	281	Gloeotrichia	-	-
796	Calothrix	+	-	602	Gloeotrichia	+	+
253	Calothrix	+	+	613	Gloeotrichia	-	+
624	Calothrix	+	+	627	Lyngbya	+	-
764	Calothrix	+	+	608	Nostoc	+	+
550	Calothrix	+	+	611	Nostoc	+	+
802	Calothrix	+	+	734	Nostoc	+	+
254	Calothrix	+	+	614	Nostoc	+	+
795	Calothrix	+	-	800	Nostoc	-	+
690	Calothrix	+	-	201	Nostoc	-	-
730	Calothrix	+	+	767	Synechococcus	+	+
266	Calothrix	+	+	807	Synechococcus	+	-
786	Calothrix	-	-	769	Synechococcus	+	+
603	Calothrix	+	+	838	Synechococcus	+	-
688	Calothrix	-	-	562	Synechococcus	+	+
202	Calothrix	+	+	33	Synechococcus	+	+
694	Calothrix	-	+	797	Synechococcus	+	+
585	Tolypothrix	-	+				

+ = staining detected after 15 min.
- = staining not detected after 15 min.

CHAPTER 4

HAIR FORMATION AND ALKALINE PHOSPHATASE ACTIVITY IN *Calothrix* 202, 550 and 603

4.1 EFFECT OF NINE PHOSPHORUS SOURCES ON YIELD, HAIR FORMATION AND ALKALINE PHOSPHATASE ACTIVITY IN *Calothrix* 202, 550 and 603

4.11 Introduction

In Chapter 3 the hair-forming Rivulariaceae strains were shown to have the highest levels of cell-bound PMEase activity at pH 10.3 and overall these strains were very effective at utilizing various organic P sources. The aims of these experiments were to investigate further if there was any correlation between trichome structure, hair formation and alkaline phosphatase activity (APA), the utilization of various organic P sources and the effects of organic P sources on APA synthesis (1.646) in *Calothrix* 202, 550 and 603. The reasons for choosing these particular cyanobacteria are explained in 4.111.

4.111 Origin and morphology of *Calothrix* 202, 550 and 603

Three cyanobacterial strains were chosen for the following studies:

Calothrix sp. 202:

This strain originates from a freshwater pool in Aldabra and never forms hairs in the field or in the laboratory. This species was chosen to act as a control to hair-forming Rivulariaceae.

Calothrix parietina 550:

This species forms very striking hairs (Sinclair, 1977) in response to various elemental deficiencies as demonstrated by Sinclair and Whitton (1977).

Calothrix sp. 603:

This strain originated from the stem of a deepwater rice plant in Bangladesh and forms hairs in the field but never formed hairs under various laboratory conditions.

4.12 Method

Growth in batch culture was investigated using eight different P-sources. Inocula were prepared (2.55) and all cultures were grown in Chu 10D-N (2.531), for 36 d at 32°C and 100 μmol photon m^{-2} s^{-1} and P was 1 mg l^{-1}.. Yield was investigated either by dry wt or by observation (2.57), samples being harvested every four d.

Table 4.1 Phosphorus sources and method used for measurement of yield.

P source	method
Potassium dihydrogen orthophosphate	Dry weight
Sodium-β-glycerophosphate	Dry weight
ρ-nitrophenyl phosphate (ρNPP)	Dry weight
2-methoxy-4-(2′-nitrovinyl)-phenyl phosphate (MNP)	Dry weight
bis (ρ-nitrophenyl) phosphate (bis-ρNPP)	Dry weight
Phytic acid	visual
DNA	visual
ATP	visual

Observation of yield was scored between 0-5 every four d. Minimum growth, i.e. in -P, was scored 0 and maximum growth in P-PO_4 was scored 5. Death in batch culture was represented as -. After 36 d the dry wt was determined (Table 4.2).

4.121 Analysis of cellular and media phosphorus

From samples harvested every four d a 25 ml algal sample was prepared for dry wt (2.57). After dry wt determination the algae were stored in a desiccated state for TFP analysis (2.6). Media samples (25 ml) were also collected at four-d intervals (2.441) for TFP analysis.

4.122 Analysis of alkaline phosphatase activity

Cell-bound and extracellular PMEase and PDEase activities were determined at four-d intervals for 36 d using ρNPP, bis-ρNPP and MNP as substrates (2.441 and 2.442). MNP was a new substrate on the market and was incorporated in a comparative study with ρNPP. MNP and ρNPP are hydrolyzed by PMEases and bis-ρNPP is hydrolyzed by PDEases.

4.13 Results

4.131 Dry weight

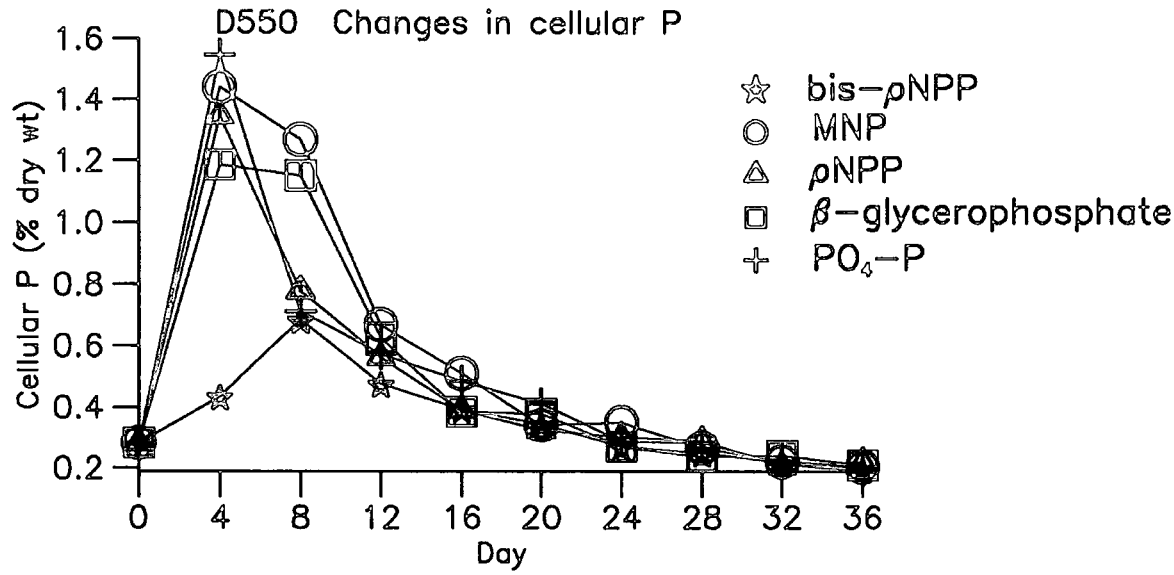
The yields were similar in Calothrix 550 and 603. However in Calothrix 202 there were enhanced yields in β-glycerophosphate and MNP and a lower yield in bis-ρNPP. Calothrix 202 and 603 showed no lag in growth. Stationary growth was at 32 or 36 d (Fig. 4.1).

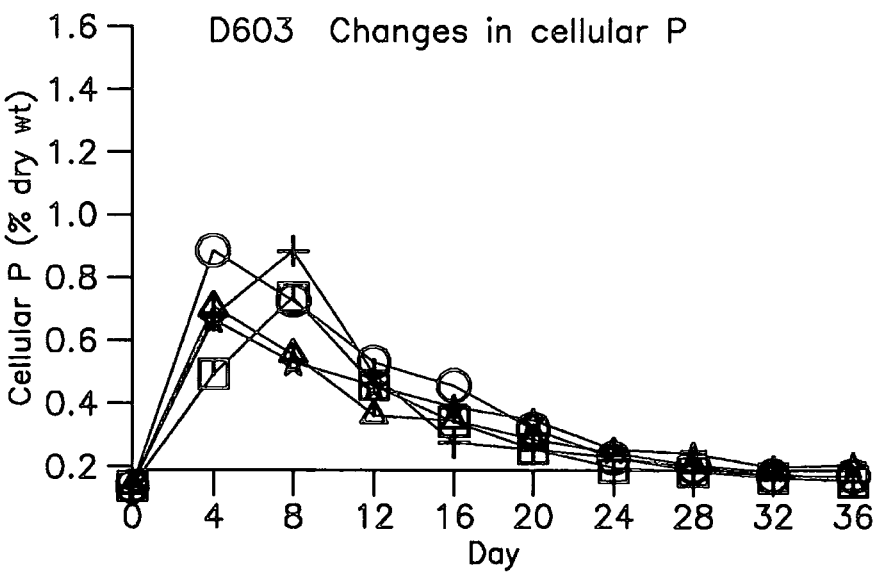
4.132 Observation of growth

Yields were reduced, with the lowest yields in phytic acid and ATP. In Calothrix 550 there was no growth in ATP and after eight d (Table 4.2) the cultures were bleached. In Calothrix 202 and 603 there was no induction of

hair formation in any P source. At eight d there were distinct differences in %hairiness in different P-sources in Calothrix 550. Hairs produced in $P-PO_4$ were 75-100 µm long and %hairiness = 5; ρNPP hairs were 75-500 µm and %hairiness = 20; there were similar observations in $P-PO_4$ and β-glycerophosphate; greatest hair formation was in bis-ρNPP, hairs were 100-500 µm long and %hairiness = 50 and in MNP hairs were 20 µm long and %hairiness = 3.

Fig. 4.1 Utilization of P-PO_4, β-glycerophosphate, ρNPP, MNP, bis-ρNPP by Calothrix 202, 550 and 603. Cultures were grown at 32°C and 100 μmol photon m^{-2} s^{-1}.





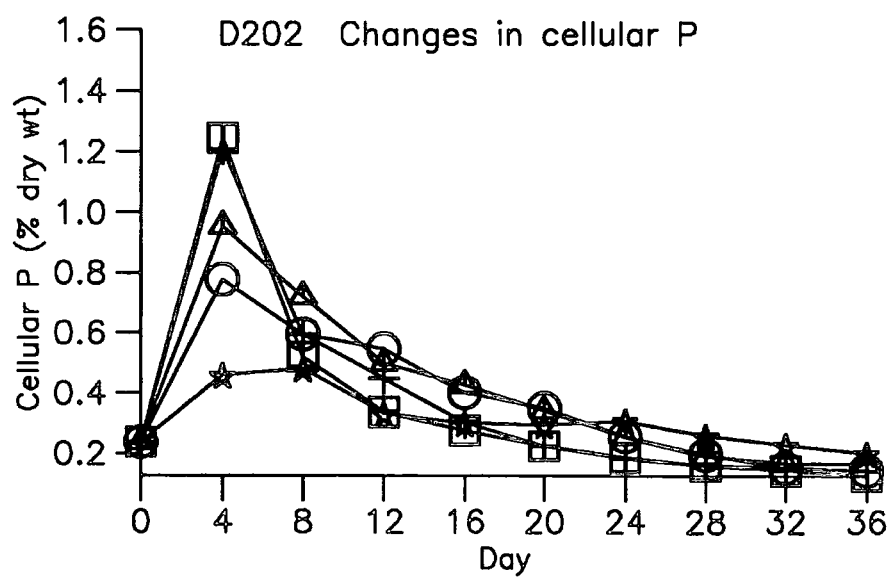


Table 4.2 Utilization of phytic acid (A), DNA (B) and ATP (C) by Calothrix 202, 550 and 603 at 32°C and 100 µmol photon m^{-2} s^{-1}.

P-source		4	8	12	16	20	24	28	32	36 (day) dry wt l^{-1}
A	550	5	2	2	2	2	2	2	3	352.50
A	603	4	4	3	3	4	4	4	4	559.75
A	202	4	3	2	1	2	3	3	3	199.75
B	550	4	4	4	5	5	5	5	5	592.50
B	603	4	4	4	4	5	5	5	5	644.75
B	202	3	4	4	3	4	4	5	5	701.50
C	550	0	0	-	-	-	-	-	-	-
C	603	3	4	4	4	5	5	5	5	630.25
C	202	0	0	0	1	2	3	3	5	614.25

KEY

- = Death of alga

0 = 0% growth or less than 0% growth

1 = 0-20% growth

2 = 20-40% growth

3 = 40-60% growth

4 = 60-80% growth

5 = 80-100% growth

0% growth is represented as growth in media -P and 100% growth is growth in P-PO_4.

4.133 Cellular phosphorus

The highest cellular P was 1.6% in Calothrix 550 at day 4. After four d there was a rapid decline in cellular P. The lowest cellular P values were in bis-ρNPP (Fig. 4.2).

4.134 Alkaline phosphatase activity

(1) Cell-bound PMEase activity, ρNPP assay (Fig. 4.3)

Growth in bis-ρNPP led to increased activity at four d in Calothrix 202 and 550. Lowest activities were in P-PO_4. In each species there were marked rises in activities after day 4, with a corresponding reduction in activity noted between days 24 - 32.

(2) Extracellular PMEase activity, ρNPP assay (Fig. 4.4)

Extracellular activities in Calothrix 550 and 603 were lower than cell-bound activities. The same pattern of increased activity in bis-ρNPP and lowest activity in P-PO_4 was noted. There was no detectable extracellular activity in Calothrix 202.

(3) Cell-bound and extracellular PMEase activity, MNP assay (Fig. 4.5 and 4.6)

The patterns of activities were the same as in the ρNPP assays, but the levels of P hydrolyzed were 15 fold greater. This suggests that MNP was more sensitive than ρNPP for assaying PMEase activity. The increased sensitivity was an advantage for detecting the lower levels of extracellular PMEase activity. Although the major drawback with MNP was that above pH 9.5 the chromatophore 2-methoxy-4-(2-nitrovinyl)-phenol was irreversibly degraded. Therefore, using MNP for assaying PMEases, with a pH optimum above 9.5 was inadvisable. However, alkaline PMEase activity in heterotrophic bacteria rarely has an optimum above pH 9.0 (1.62).

(4) Cell-bound PDEase activity, bis-ρNPP assay (Fig. 4.7)

The highest levels of PDEase activity were in Calothrix 603. There were no differences in activities between the five P-sources for each species. In Calothrix 202 and 550 highest activities were at day four and day eight respectively.

(5) Extracellular PDEase activity, bis-ρNPP assay (Fig. 4.8)

The only species which exhibited extracellular PDEase activity was Calothrix 603. The levels of activities were low.

Fig. 4.2 Change in cellular P status with time in Calothrix 202, 550 and 603. P-sources used were P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP. Cultures were grown at 32°C and 100 μmol photon m^{-2} s^{-1}.

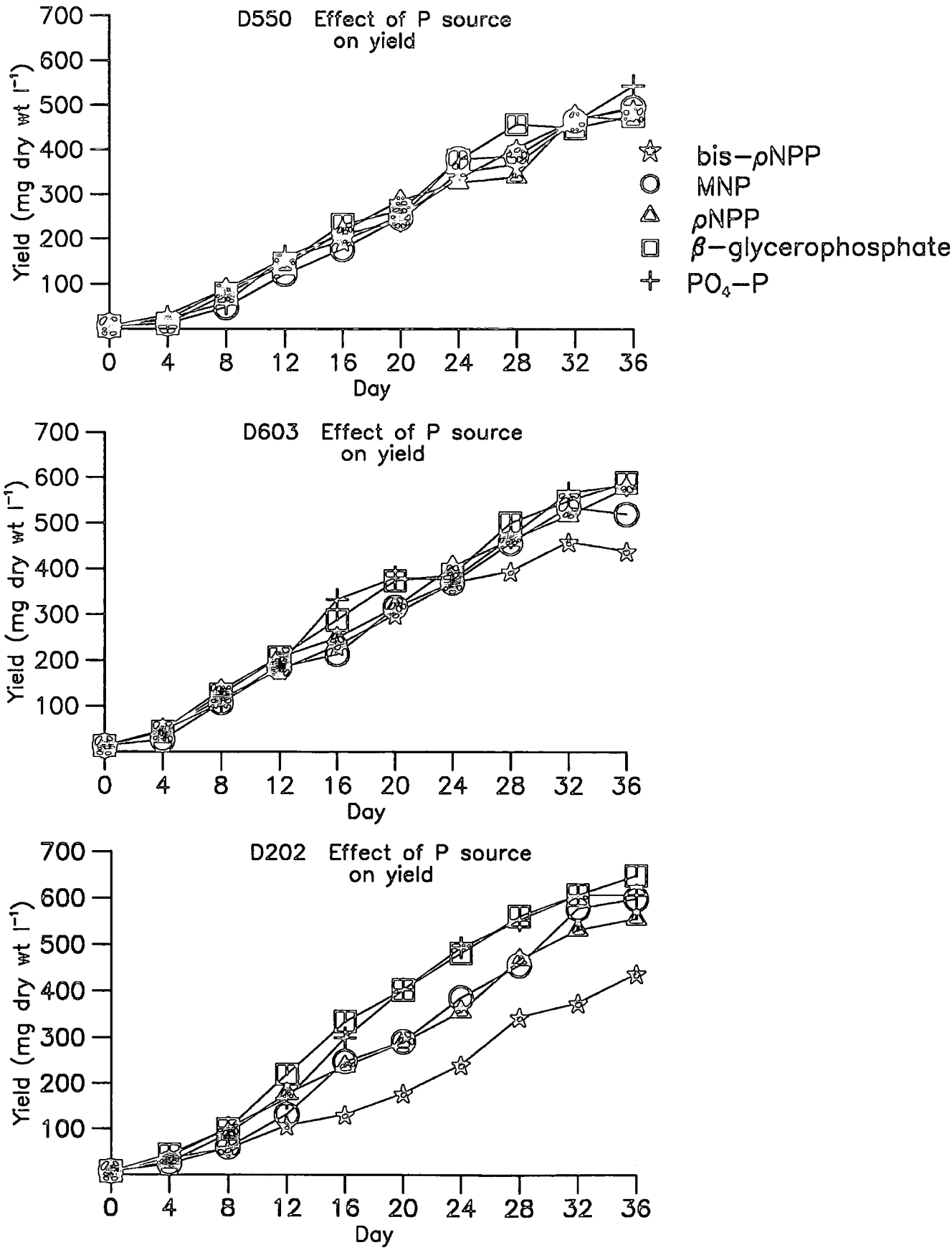


Fig. 4.3 Cell-bound PMEase activity in Calothrix 202, 550 and 603 assayed at 32°C using ρNPP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 μmol photon m^{-2} s^{-1}.

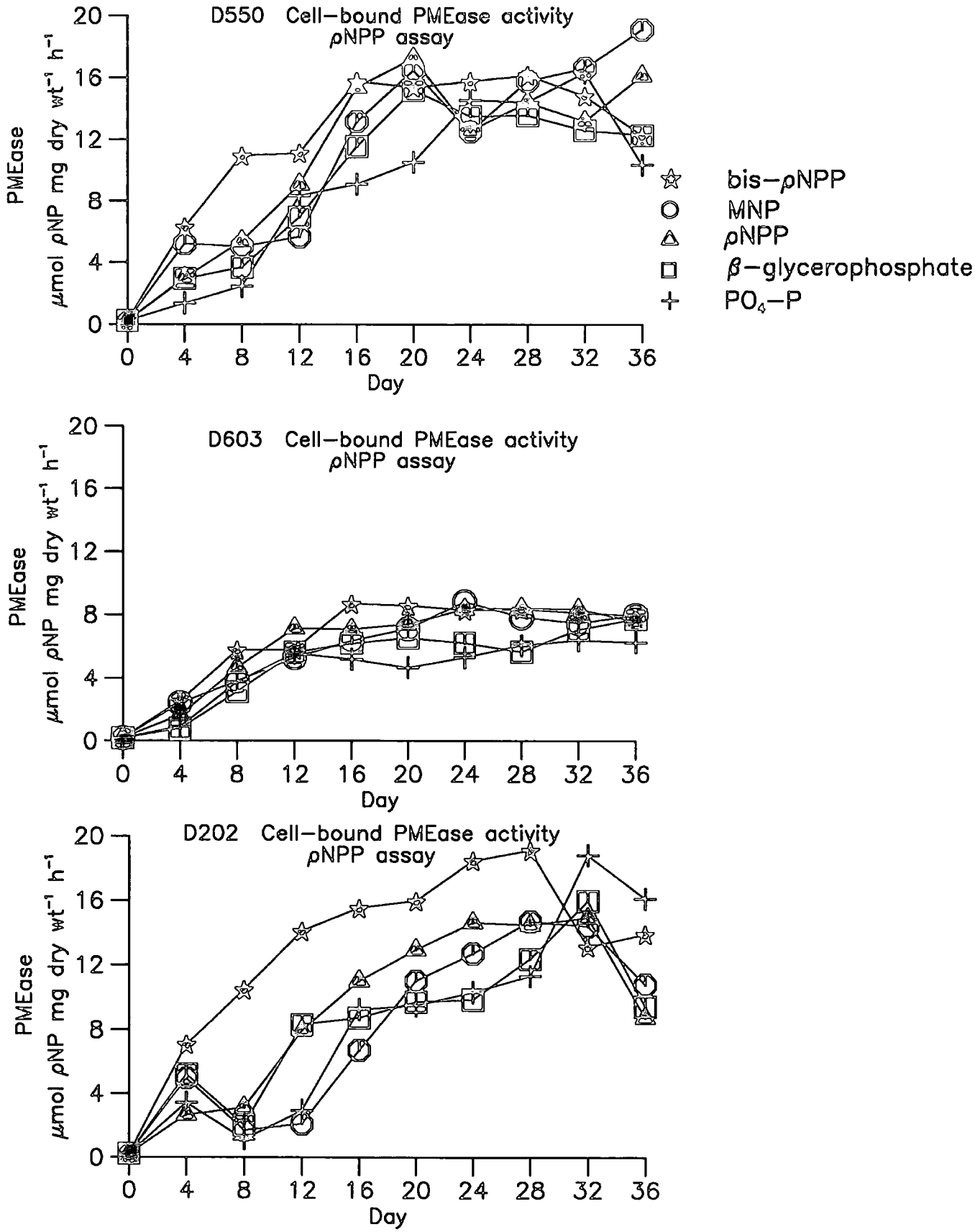


Fig. 4.4 Extracellular PMEase activity in Calothrix 550 and 603 assayed at 32°C using ρNPP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 μmol photon m^{-2} s^{-1}.

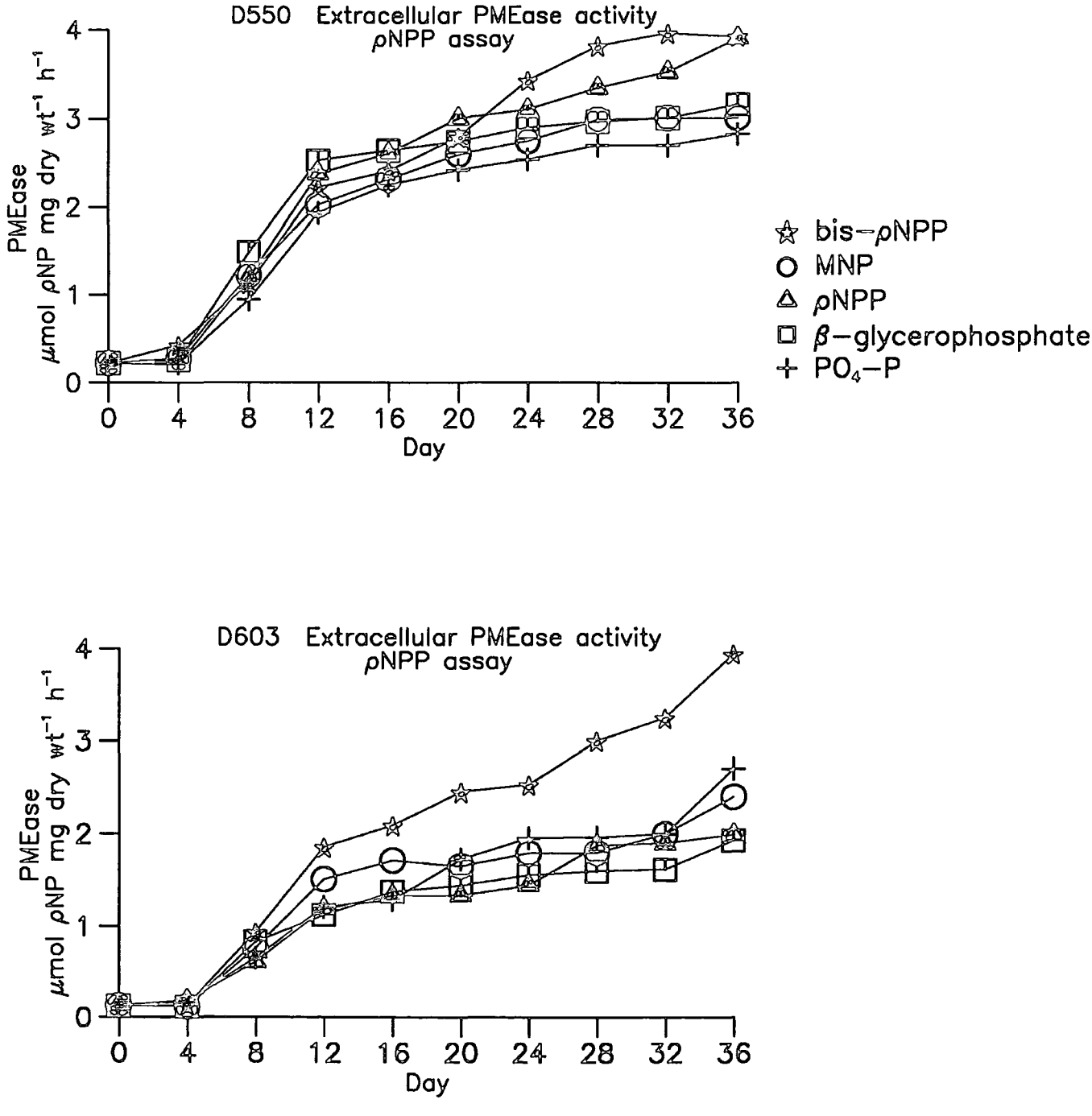


Fig. 4.5 Cell-bound PMEase activity in Calothrix 202, 550 and 603 assayed at 32°C using MNP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 µmol photon m^{-2} s^{-1}.

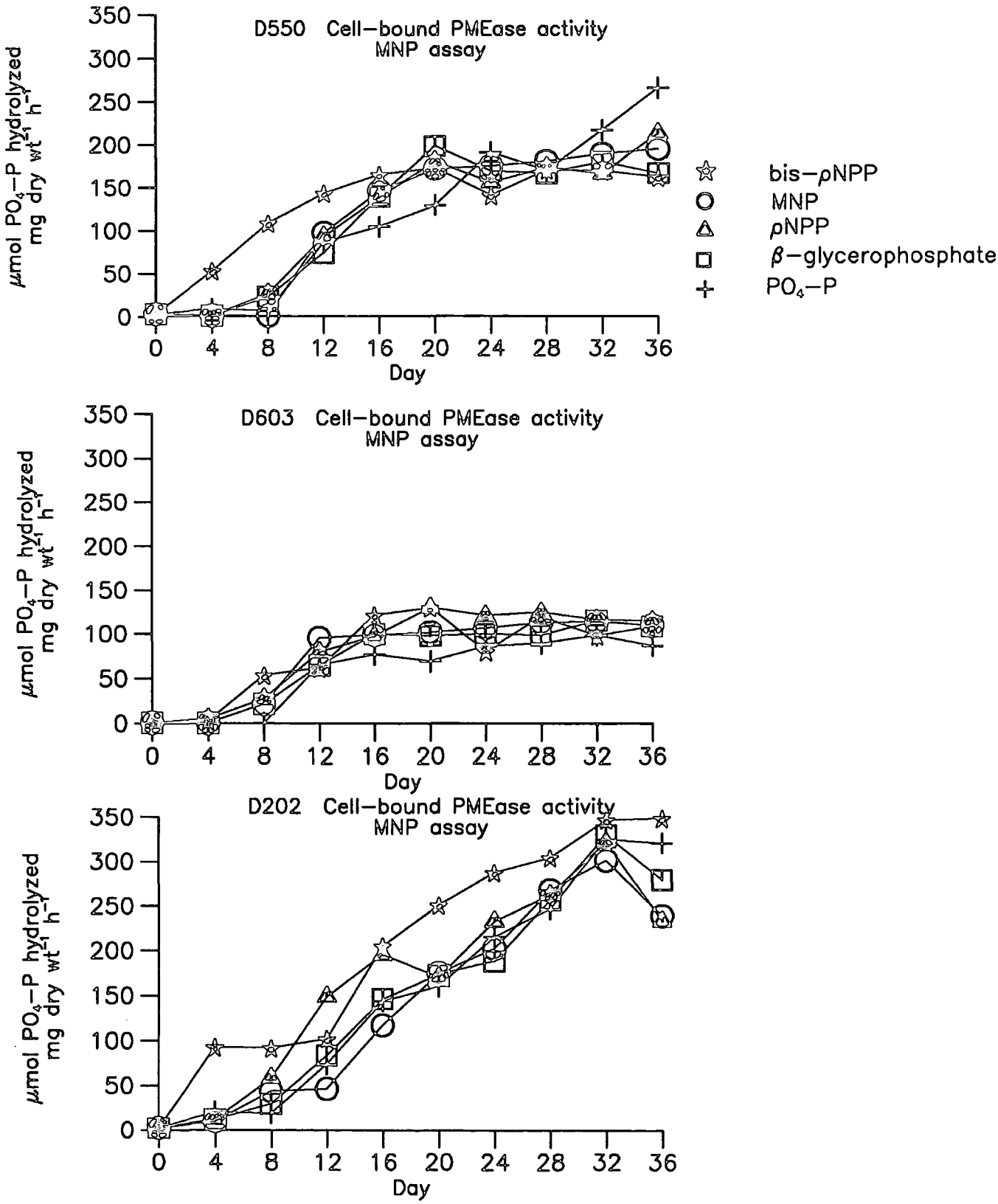
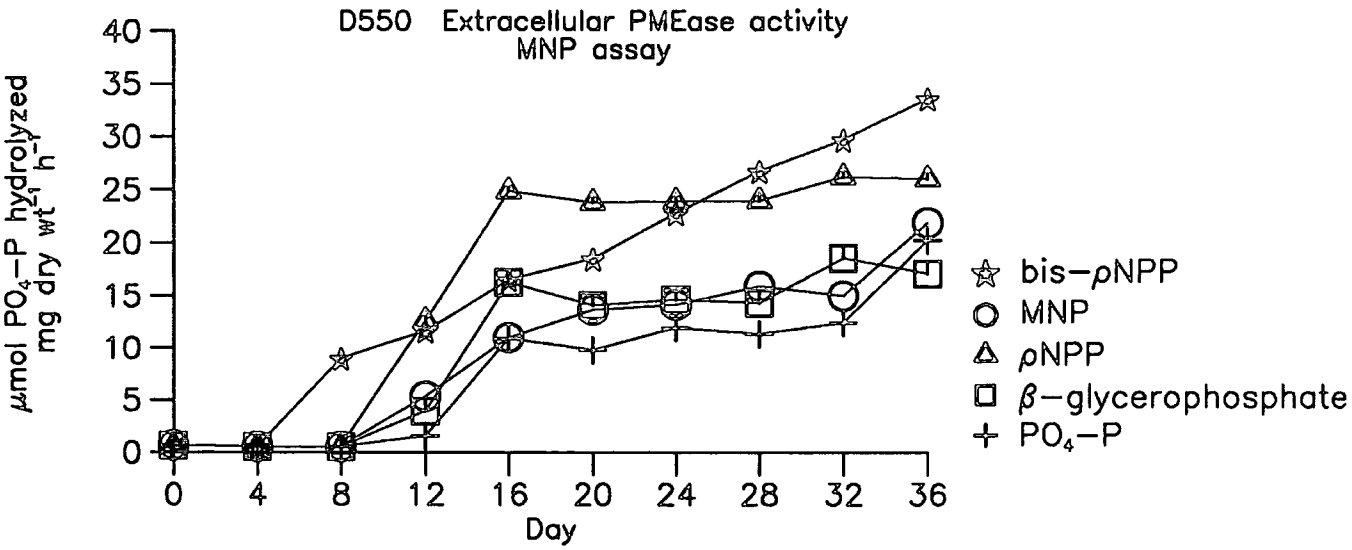


Fig. 4.6 Extracellular PMEase activity in Calothrix 550 and 603 assayed at 32°C using MNP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 μmol photon m^{-2} s^{-1}. There was no detectable activity in Calothrix 202.



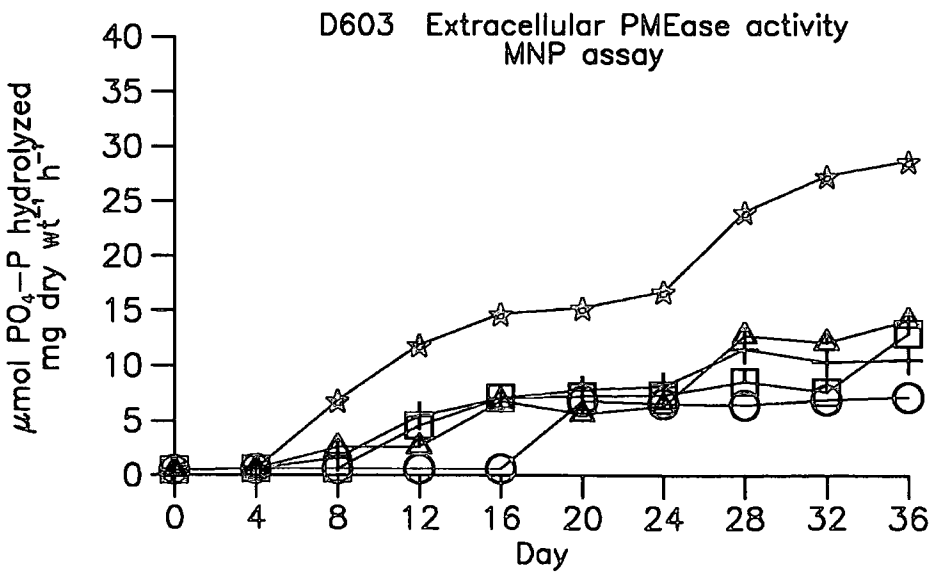
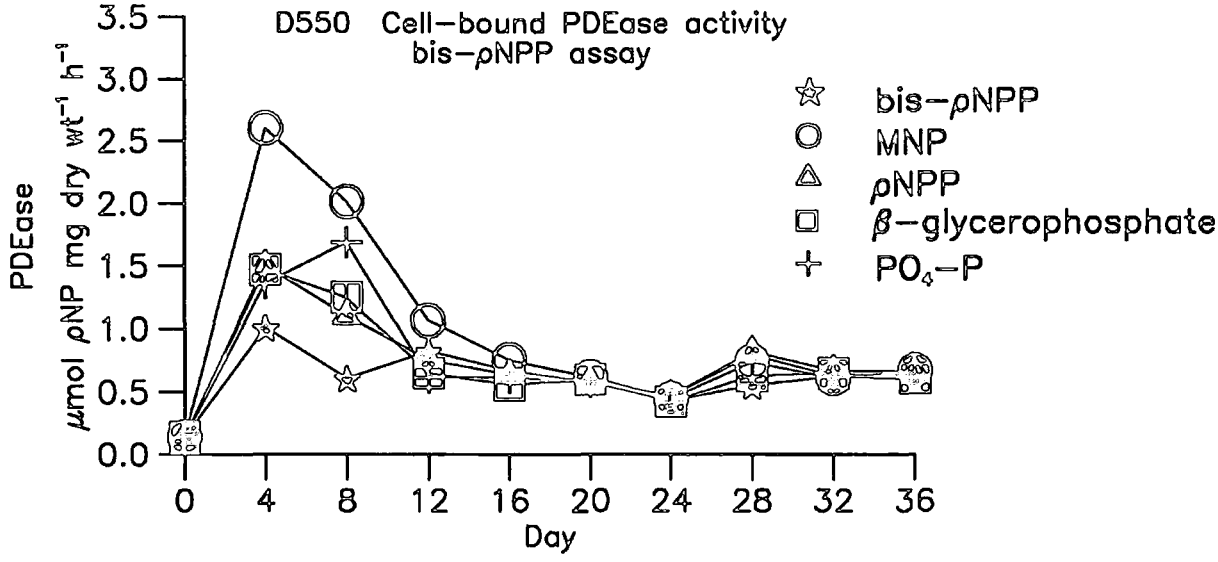
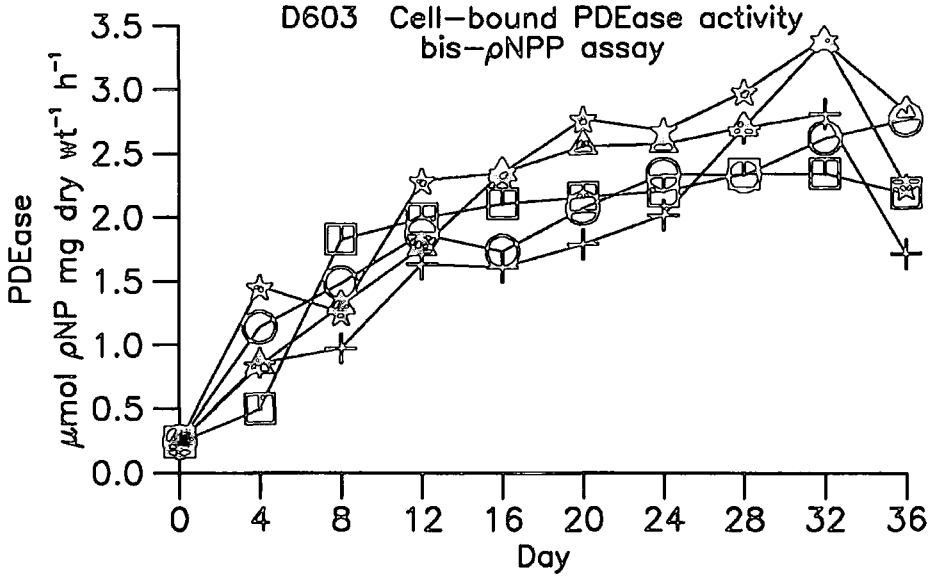


Fig. 4.7 Cell-bound PDEase activity in Calothrix 202, 550 and 603 assayed at 32°C using bis-ρNPP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 μmol photon m^{-2} s^{-1}.





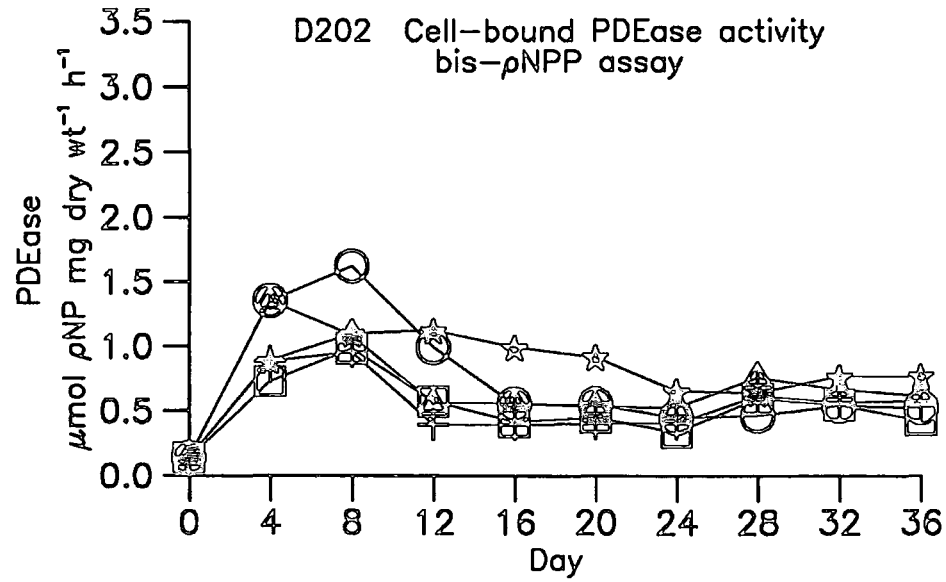
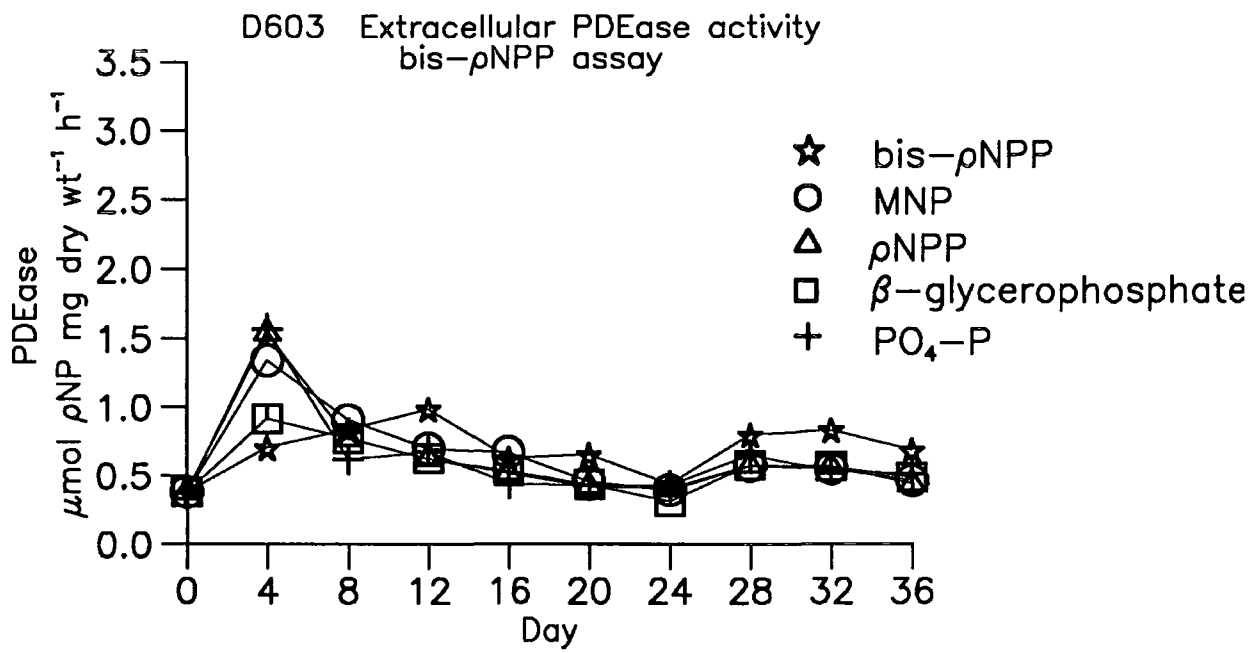


Fig. 4.8 Extracellular PDEase activity in Calothrix 603 assayed at 32°C using bis-ρNPP. Cultures were grown in 5 P-sources P-PO_4, β-glycerophosphate, ρNPP, MNP and bis-ρNPP at 32°C and 100 μmol photon m^{-2} s^{-1}. There was no detectable activity in Calothrix 202 and 550.



4.2 INFLUENCE OF pH ON PHOSPHATASE ACTIVITY IN Calothrix 202, 550 and 603

4.21 Introduction

In 4.1 no major differences in the utilization of organic P sources were shown between Calothrix 202, 550 and 603. However, in Chapter 3 one major difference between hair-forming and non hair-forming Rivulariaceae was the effect of pH on PMEase activity. Hair-forming Rivulariaceae had greater activity at pH 10.3 than pH 7.6, whereas there was little difference in activity in non hair-forming Rivulariaceae. Therefore, it was decided to see if there were differences in the influence of pH and to see if acid phosphatases were present (1.641). Differences or similarities in the influence of pH on activity of cell-bound and extracellular PMEases and PDEases in Calothrix 202, 550 and 603 may suggest that the fractions have phosphatases of a common origin.

4.22 Method

To determine the effects of pH, assays were carried out between pH 3-11, using duplicate buffers at each pH value (2.45 and Table 2.3). The algae used were 16 d old grown in Chu 10D-N, P_i 1 mg l^{-1}, at 32°C and 100 µmol photon m^{-2} s^{-1}. Algae were prepared for assays as in 2.441. Assays ran for 30 min and were terminated by the addition of 100 µl of 4.95 M NaOH. A different protocol was used for PDEase activity (2.45), because the addition of 100 µl of 4.95 M NaOH resulted in bis-pNPP hydrolysis. The levels of cell-bound and extracellular PDEase activity in Calothrix 202 and 603, respectively, were too low to accurately measure any changes due to pH.

4.23 Results

(1) Cell-bound PMEase activity (Fig. 4.9)

PMEase activities in Calothrix 202 and 550 were detectable between pH 7-11 with greatest activity at pH 10.3. In Calothrix 603 optimum activity was between pH 7 and 8.

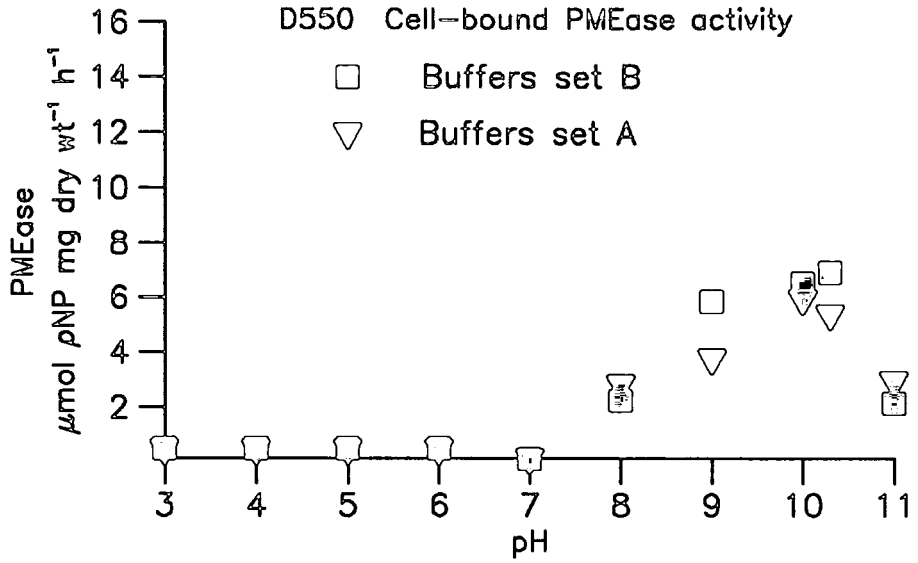
(2) Extracellular PMEase activity (Fig. 4.10)

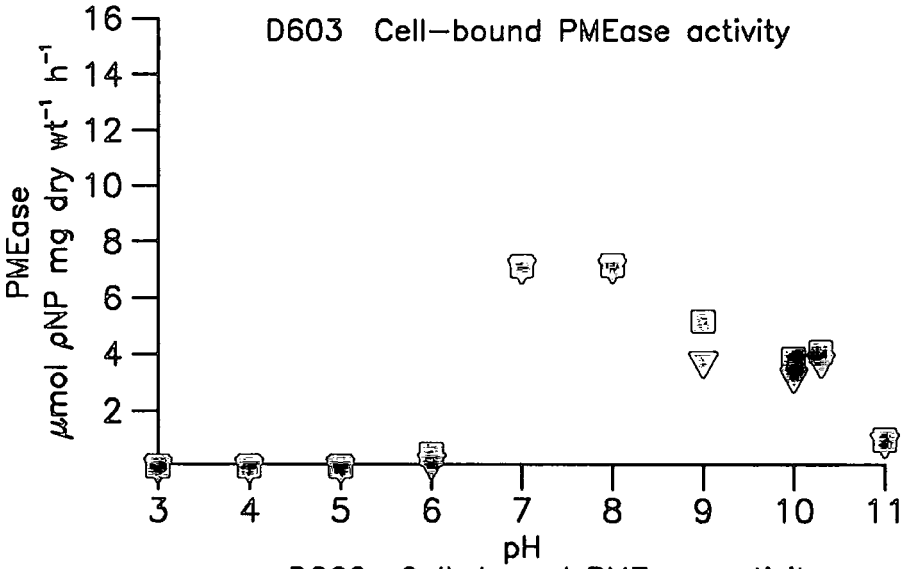
In Calothrix 550 and 603 extracellular PMEase activity was lower than cell-bound activity, although the response to pH was the same. This suggests that the PMEases in the two fractions may have a common origin. There was no detectable extracellular activity in Calothrix 202.

(3) Cell-bound PDEase activity (Fig 4.11)

The patterns of activity in Calothrix 550 and 603 were significantly different. In Calothrix 550 and 603 optimum activity was at pH 10.3 and 8 respectively. PDEase activity was overall much lower than PMEase activity at all pH values, which agrees with previous results for PDEase activity.

Fig. 4.9 Influence of pH on cell-bound PMEase activity in Calothrix 202, 550 and 603, assayed at 32°C using ρNPP. Cultures were grown in 1 mg l^{-1} $P\text{-}PO_4$ at 32°C and 100 μmol photon m^{-2} s^{-1}.





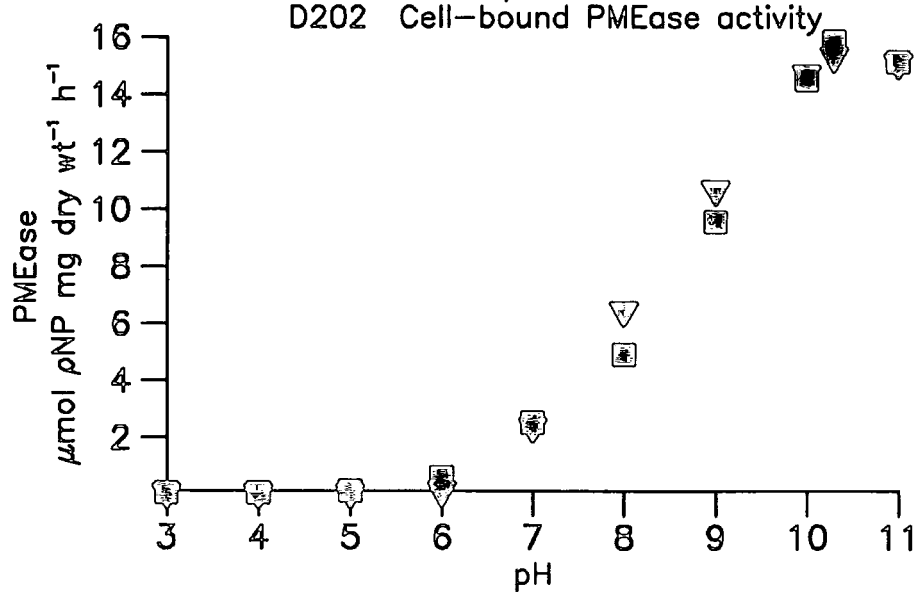
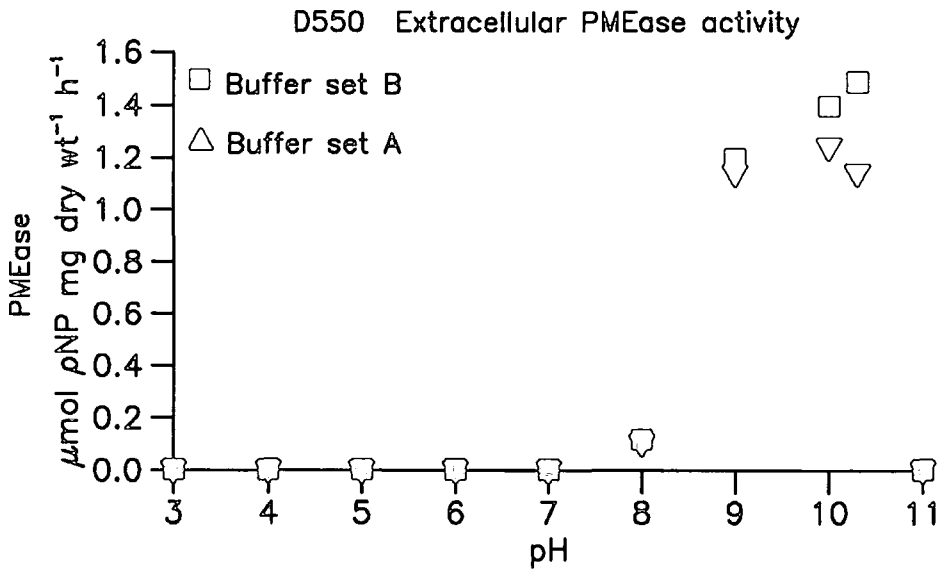


Fig. 4.10 Influence of pH on extracellular PMEase activity in Calothrix 550 and 603, assayed at 32°C using ρNPP. Cultures were grown in 1 mg l^{-1} $P-PO_4$ at 32°C and 100 μmol photon m^{-2} s^{-1}. There was no detectable extracellular activity in Calothrix 202.



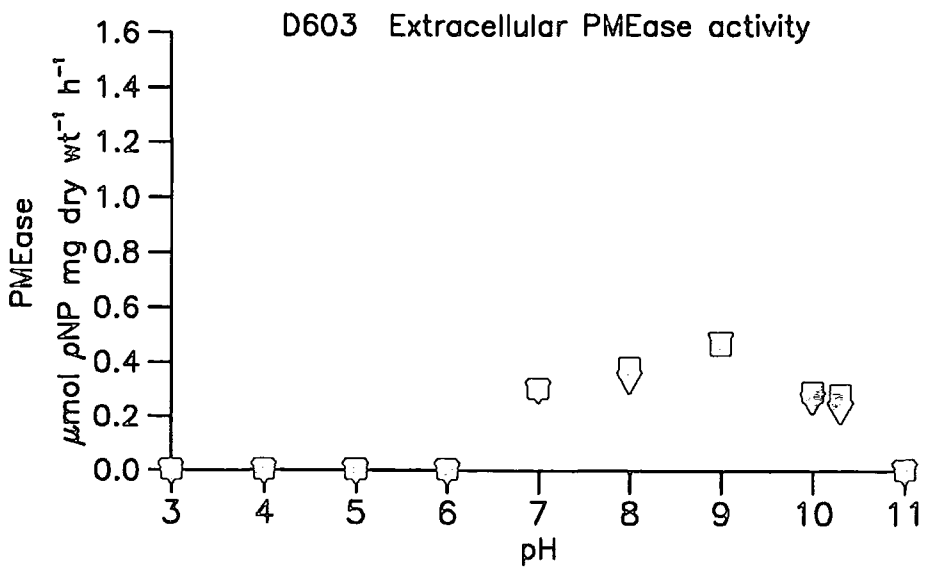
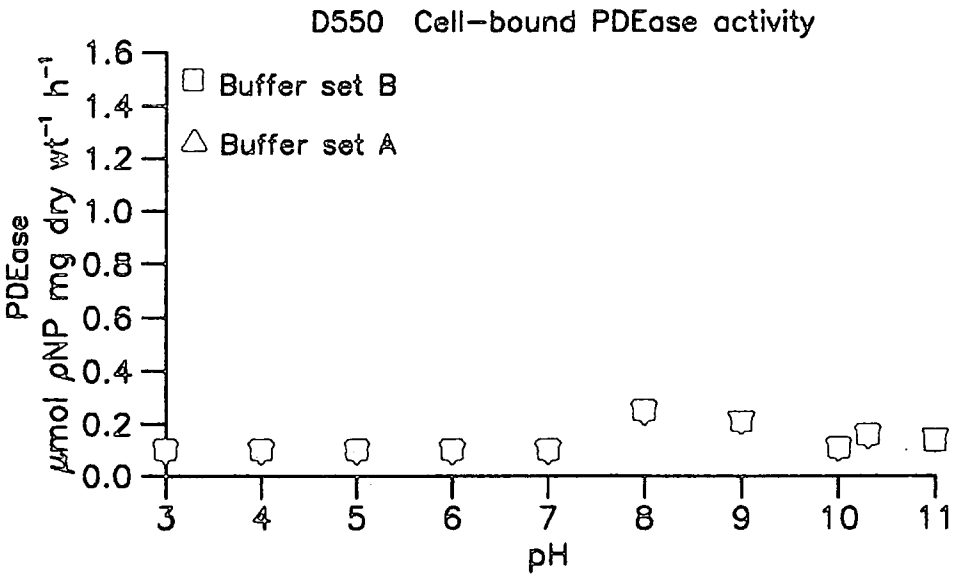
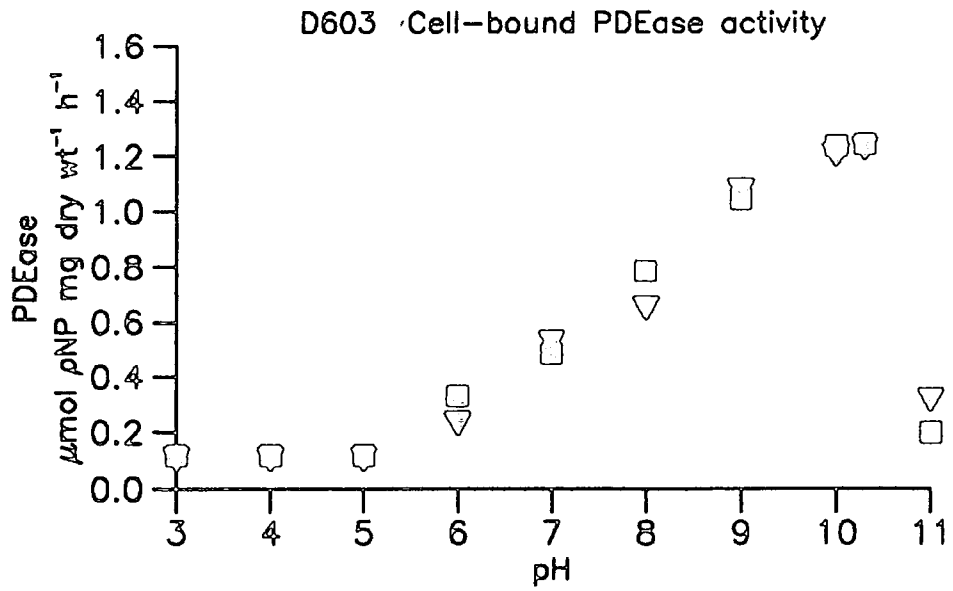


Fig. 4.11 Influence of pH on cell-bound PDEase activity in Calothrix 550 and 603, assayed at 32°C using bis-ρNPP. Cultures were grown in 1 mg l^{-1} $P\text{-}PO_4$ at 32°C and 100 µmol photon m^{-2} s^{-1}.





CHAPTER 5

INFLUENCE OF TEMPERATURE, pH, IONS AND SUBSTRATE CONCENTRATION ON CELL-BOUND AND EXTRACELLULAR PHOSPHOMONOESTERASE ACTIVITY IN *Calothrix* 550

5.1 INFLUENCE OF TEMPERATURE ON CELL-BOUND AND EXTRACELLULAR PHOSPHOMONOESTERASE ACTIVITY IN *Calothrix* 550

5.11 Introduction

Extracellular PMEase activity has been reported in varying amounts in micro-organisms (1.6). Doonan and Jensen (1980) investigated 18 cyanobacterial isolates and 10 exhibited extracellular PMEase activity. If in *Calothrix* 550 there were no significant differences in response to temperature, pH and ions for cell-bound and extracellular PMEase activity, it may suggest that the same enzyme is in the cell-bound and extracellular fraction. The aim of these experiments were to establish to what extent PMEase activities in a hair forming rivulariaceaen, *Calothrix* 550, resembled those reported in other cyanobacteria and to what extent cell-bound and extracellular PMEase activities differ in their response to environmental factors.

5.12 Method

Calothrix 550 was grown in batch culture at 25°C and 60 µmol photon m^{-2} s^{-1} in Chu 10D-N (P 1mg l^{-1}). Flasks were agitated daily to prevent CO_2 limitation. Cultures were harvested at a late growth stage (28 d) when markedly P-limited. Cellular material was obtained by centrifugation at 8000 x g for 20 min, washed twice, resuspended in assay medium (2.533) and homogenized (2.441). Further assays on extracellular activity were based on material harvested on this occasion and subsequently stored at -20°C.

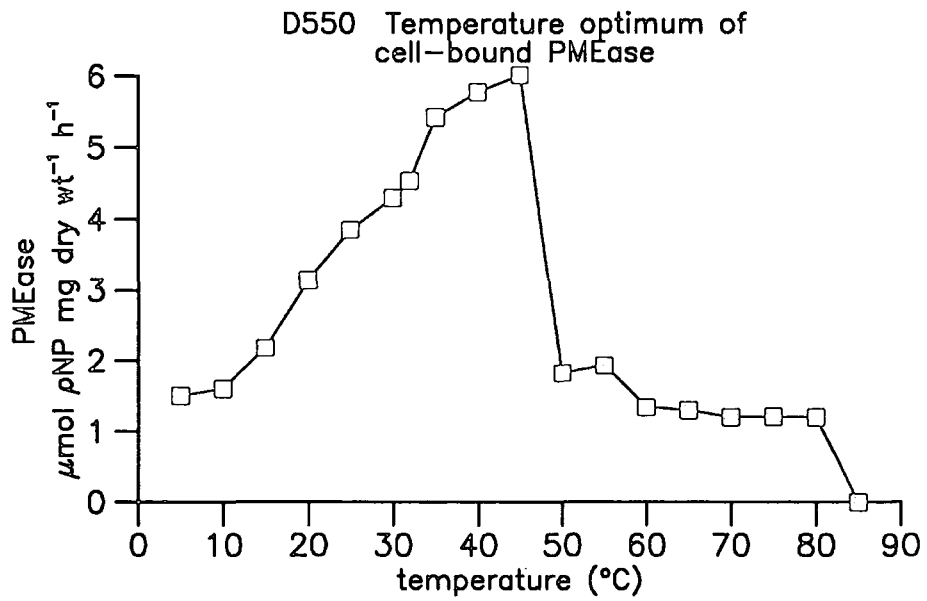
Storage over periods up to seven d at 4°C and for longer periods (up to 12 months) at -20°C led to no detectable change in activity. Three litres were rotary evaporated to 150 ml, centrifuged at 8000 x g for 20 min to remove cell debris and the supernatant dialyzed against assay medium, with three changes over 24 h.

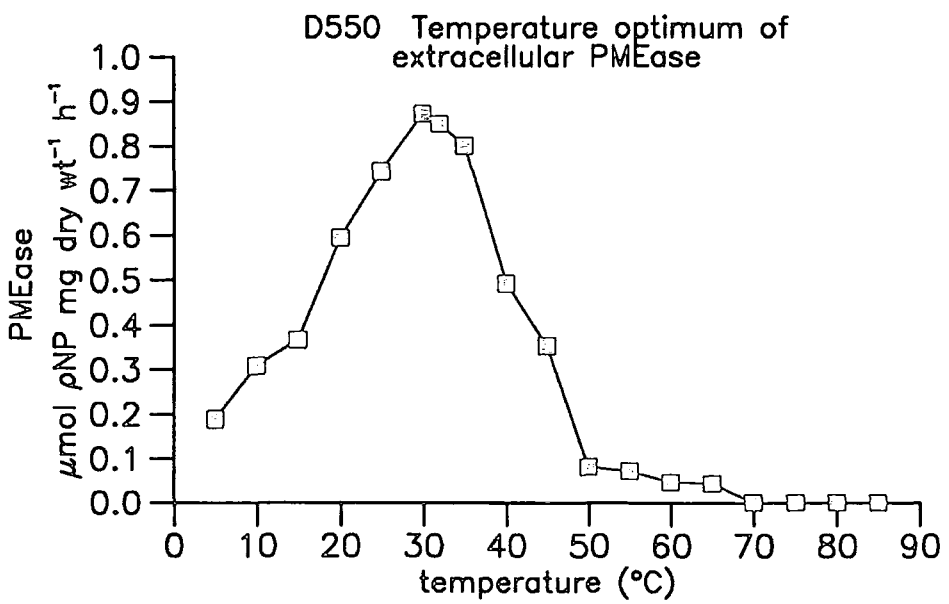
To test the effect of temperature on cell-bound and extracellular PMEase activity, aliquots of homogenate or medium were pre-incubated for 30 min at 5°C intervals between 5 and 85°C. Deviations in pH from 10.3 due to temperature were compensated by the addition of 1 M NaOH or HCL. Temperatures above 85°C could not be tested directly because of the spontaneous hydrolysis of ρNPP; the ability of the enzyme to tolerate temperatures above 85°C was tested by again lowering the temperature to 32°C and testing with ρNPP. Activity was terminated after 30 min.

5.13 Results

Temperature optima for cell-bound and extracellular PMEase activity were 45°C and 30°C, respectively, and maximum temperatures with detectable activity were at 80°C and 65°C, respectively (Fig. 5.1). Both enzyme systems were denatured above 90°C. Only PMEase activity in toluenized suspensions of E. coli are stable at 90°C (Torriani, 1960). Doonan and Jensen (1980) found a similar temperature optimum (40°C) for cell-bound PMEase activity in Plectonema boryanum. Doonan and Jensen (1980) showed that the cell free enzyme extract had a higher temperature optimum (70°C) than the cell-bound fraction, although they supplied no explanation for this occurrence. Further experiments on PMEase activity were carried out at 32°C.

Fig. 5.1 Influence of temperature on cell-bound and extracellular PMEase activity in Calothrix 550. Cultures were grown for 28 d at 25°C and 60 μmol photon m^{-2} s^{-1}.





5.2 INFLUENCE OF pH ON CELL-BOUND AND EXTRACELLULAR PHOSPHOMONOESTERASE ACTIVITY IN *Calothrix* 550

5.21 Introduction

In Chapter 4 cell-bound and extracellular PMEase activity had a similar response to pH. The exact pH optima for the two systems were needed for further proof of the similarities between the two enzyme systems.

5.22 Method

Cell-bound and extracellular PMEase activity was assayed using pNPP at 32°C (2.443). The influence of pH between pH 7.0 - 11.0 was tested using a range of buffers (Table 5.1). Two different buffers were tested at each pH value. Quite similar results were obtained with each buffer, except for pH 9.0 (glycine much higher than 2-amino-2-methyl-1-propanol) and pH 11.0 (3-cyclohexylamino-1-propanesulphonic acid much higher than Na_2CO_3 - $NaHCO_3$).

5.23 Results

There were no marked changes in activity between pH 9.8-10.2 (Fig. 5.2) and activity decreased above pH 10.2 in each enzyme system. As the exact pH optimum was not defined, the initial pH buffering system of glycine-NaOH (50 mM) pH 10.3 (SIGMA Technical Bulletin no. 104) was used for further experiments.

Table 5.1 Buffers used for testing the effect of pH on cell-bound and extracellular phosphomonoesterase activity in Calothrix 550. The buffer which led to the higher activity and was used for data in Fig. 5.2 is indicated here, unless activity was below the detection limit (< 0.02 µmol ρNPP hydrolyzed mg dry wt^{-1} h^{-1}).

Abbreviations:

DMG, 3,3-dimethylglutaric acid;

HEPES, N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid;

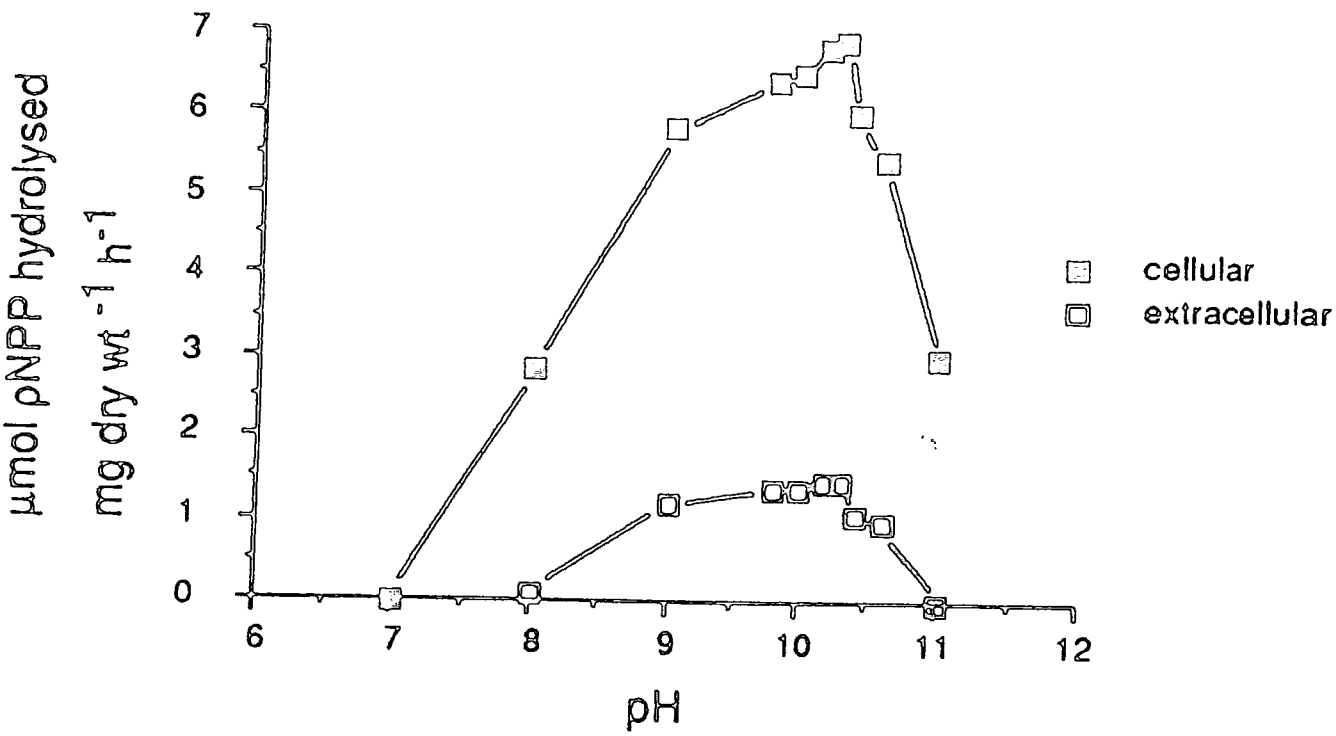
TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid;

AMeP, 2-amino-2-methyl-1-propanol;

CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid.

pH	buffer A	buffer B	buffer giving higher activity	
			cellular	extracellular
7.0	DMG - NaOH	HEPES - NaOH		
8.0	TES - NaOH	HEPES - NaOH	TES	TES
9.0	AMeP - NaOH	glycine - NaOH	glycine	glycine
9.8	AMeP - NaOH	glycine - NaOH	glycine	glycine
10.0	AMeP - NaOH	glycine - NaOH	glycine	glycine
10.2	AMeP - NaOH	glycine - NaOH	glycine	glycine
10.3	AMeP - NaOH	glycine - NaOH	glycine	glycine
10.4	AMeP - NaOH	glycine - NaOH	glycine	AMeP
10.6	AMeP - NaOH	glycine - NaOH	AMeP	AMeP
11.0	CAPS - NaOH	Na_2CO_3 - $NaHCO_3$	CAPS	

Fig. 5.2 Influence of pH (7-11) on cell-bound and extracellular PMEase activity in Calothrix 550. Cultures were grown for 28 d at 25°C and 60 μmol m^{-2} s^{-1}.



5.3 INFLUENCE OF IONIC COMPOSITION, LOCALIZATION AND PARTIAL PURIFICATION OF PHOSPHOMONOESTERASE ACTIVITY IN *Calothrix* 550

5.31 Introduction

The function of alkaline phosphatases with regard to their requirements for particular ions varies in different micro-organisms. If cell-bound and extracellular PMEase activity in *Calothrix* 550 had a similar response to different ions it may suggest that each system contained the same enzyme. Only one other study by Doonan and Jensen (1979) for *Plectonema boryanum* has investigated the effect of ions, over a range of concentrations, on PMEase activity. In Chapter 3 localization of cell-bound PMEase activity in 51 cyanobacterial strains was carried out. Further localization of cell-bound PMEase activity may conclude that hairs in *Calothrix parietina* contain a large proportion of the phosphatase activity. Purification of extracellular PMEase activity, using biochemical protein extractions and electrophoretic analysis of the proteins, would eventually lead to conclusive proof using immuno-cytochemical localization that PMEase activity was located on and/or in hair cells. Extracellular PMEase fraction was used for purification, as previous experiments (4.23, 5.1 and 5.2) showed there were no significant differences between cell-bound and extracellular PMEase activities and it was likely that the same PMEase or PMEases were present in both systems (Grainger *et al.*, 1989).

5.32 Method

Cell-bound and extracellular PMEase activities were assayed using ρNPP at pH 10.3 (glycine-NaOH, 50 mM) and 32°C. Cellular material was harvested as in 5.12. The assays were carried out as in 2.443 and enzyme activity was terminated after 15 min. The ions were dissolved in ρNPP. The range of concentrations was 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM. The ions

tested were Ca^{++}, Mg^{++}, Co^{++}, FeIII-chelate, Zn^{++}, CuII, Na^{+}, K^{+}, phosphate, borate and molybdate. Cations were added as the relevant chloride or sulphate, which have no reported effect on PMEase activity; NaOH used for buffering glycine was replaced by KOH in the case of the Na assay. Anions were added as the sodium salt.

The influence of EDTA on PMEase activity was tested in two ways: its inclusion during an assay (medium, substrate and EDTA at 0.09, 1, 10 and 20 mM) and by washing followed by resuspension in assay medium. For the latter, material was harvested (5.12), resuspended in medium or medium with increased EDTA (1, 10 or 20 mM) for 30 min, washed twice again to remove EDTA and then assayed for PMEase activity (in medium). The presence of 20 mM EDTA reduced the pH of assay medium to 9.78. The effect of sodium dodecyl sulphate (SDS) and NaOH on cell-bound and extracellular PMEase activity was also tested.

A fraction consisting almost entirely of detached hairs was obtained by sonicating a washed culture for five s, centrifuging at a low speed (500 x g) for 10 min and retaining the supernatant. The supernatant was then centrifuged at 8000 x g for 20 min and the pellet retained. At least 95% of the contents of the pellet consisted of hairs. This fraction was used to test the ability of hairs to hydrolyze ρNPP, bis-ρNPP, BCIP (2.731) and naphthol AS-MX phosphate (2.732).

Carbohydrate in medium from 28-d cultures was assayed by the method of Dubois et al. (1956), in order to establish whether there was a correlation between extracellular PMEase activity and carbohydrate (2.62). Localization of enzyme activity was studied by the use of various methods likely to release PMEase: trichloroethane; 20% sucrose; lysozyme-treated material previously exposed to 20% sucrose (Ingram et al., 1973). Localization was also tested by microscopy using BCIP (2.731) as an organic P substrate.

Carbohydrates were also localized on gels using danzyl hydrazine. Gels were fixed overnight in 40% ethanol 5% acetic acid, all solutions were diluted

in MilliQ water. Gels were oxidized for two h[ovs] in 5% acetic acid and 0.7% periodic acid and then washed in MilliQ water. Periodic acid was removed with 0.5% sodium meta-bisulphite in 5% acetic acid for one h, gels were washed twice in MilliQ. Gels were then stained with 600 µl of concentrated HCL in one litre of dimethyl sulphoxide (DMSO) containing two mg ml^{-1} danzyl hydrazine. This was incubated at 60°C for two h. Five ml of 0.2 mg $NaBH_4$ ml^{-1} DMSO was added and incubated at room temp for 30 min. The gel was rinsed with MilliQ water and destained overnight in 1% acetic acid. Gels were illuminated at 366 nm (UV light) to detect carbohydrate.

Further purification of extracellular PMEase in Calothrix 550 was carried out using the extracellular fraction from 5.12, which was the most suitable fraction for purification. This extracellular PMEase fraction was ultracentrifuged at 110,000 x g for one h and then two ml aliquots of the supernatant and pellet (resuspended in an equivalent volume of assay medium) were vacuum centrifuged to 50 µl. 50 µl samples were electrophoresed on non-denaturing polyacrylamide gels and stained with BCIP. Filter paper saturated with 1 mM BCIP (2.731) was positioned on top of the gel. Gels were sealed in a polyethene bag and left for c. one h. Protein bands containing active PMEase were located as discrete blue bands on the gel.

Protein bands located with BCIP were cut out, placed in 2 ml of assay medium (2.532), homogenised with a polytron for 5 min, sonicated for 15 min, centrifuged at 5000 x g and the supernatant assayed for PMEase activity using ρNPP at pH 10.3 and 32°C (2.45). This procedure would release PMEase if physically attached to the gel.

Column chromatography, using various ion-exchange matrices; acetone precipitation of proteins (Ahmed and King, 1960); ammonium sulphate and butanol extraction of proteins (Morton, 1950) combined with electrophoresis of denaturing (5 - 10% SDS) and non-denaturing polyacrylamide and agarose gels using extracellular and cellular material harvested at different stages of

growth, which are standard methods used in extraction of PMEases (McComb et al., 1979), did not produce discrete protein bands.

5.33 Results

The responses of cell-bound and extracellular PMEase are depicted in Fig. 5.3. Calcium had the greatest stimulatory effect, with a 50% increase in activity at 10 mM for both systems (Fig. 5.3). 0.01 mM K^+ increased extracellular activity (Fig. 5.3) and a lack of Na^+ enhanced extracellular activity (Fig. 5.3). Zinc stimulated extracellular activity up to 0.1 mM and inhibited activity at higher concentrations (Fig. 5.3). Molybdate had a slight inhibitory effect at 1 mM and a marked inhibitory effect at 10 mM; magnesium and borate also had marked inhibitory effects at 10 mM. Phosphate at 0.01 mM had no inhibitory effect, but in the range 0.1 - 10 mM it had the greatest effect of any ion (Fig. 5.3). Iron, copper and cobalt showed little effect on activity (Fig. 5.3).

The presence of EDTA at concentrations of 1 mM and above in assay medium led to complete inhibition of PMEase activity (Table 5.2). However when filaments, which had been suspended in EDTA solutions of the same molarity, were resuspended in normal assay medium, there was only a slight decrease in PMEase activity (Table 5.2).

A higher concentration of NaOH was required to terminate cell-bound than extracellular activity. 0.3 M NaOH terminated extracellular activity, but reduced cell-bound activity by only 85%. 1.5 M NaOH was required to terminate the latter effectively. 1% SDS inhibited extracellular activity completely, but reduced cell-bound activity by only 20%.

Table 5.2 Effect of EDTA on cell-bound phosphomonoesterase activity in Calothrix 550 during and prior to assay; n = 8. (The slight decrease in activity associated with the pH drop at 20 mM EDTA is negligible).

EDTA (mM)	0.09		1		10		20	
	μmol ρNPP hydrolyzed mg dry wt^{-1} h^{-1}							
treatment	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
during assay	5.82	0.27	<0.02		<0.02		<0.02	
prior to assay	6.02	0.08	5.75	0.64	5.18	1.07	4.64	0.34

Evidence that hairs are an important site of enzyme activity was obtained by use of a cellular fraction consisting of detached hairs (5.32). This fraction showed very high activity with ρNPP, bis-ρNPP and BCIP, although the mass of hairs available was too low to obtain a rate. There was no detectable activity with naphthol AS-MX phosphate when observed under the microscope.

Several tests were carried out to establish whether cell-bound enzyme was attached to a surface (such as cell wall or plasma membrane) or present in the periplasmic space. No significant increase in extracellular activity occurred when cell-bound material was exposed to trichloroethane (0.1 mM - 100 mM), 20% sucrose or lysozyme treatment. These all suggest that the enzyme is bound to a surface.

It seemed possible that extracellular PMEase may be bound to colloidal carbohydrate material related to sheath carbohydrates (Weckesser et al., 1988) Ultracentrifugation (110,000 x g) of the extracellular PMEase fraction (5.12) for 1 h removed 80% of the carbohydrate from the supernatant, but only 45% of enzyme activity, suggesting that at least part of the extracellular enzyme is possibly soluble.

Four distinct blue bands (Fig 5.4a,) using BCIP were detected on a non-denaturing polyacrylamide gel using the pellet from 110,000 x g centrifugation

and one PMEase band was located in the supernatant. However, these PMEase bands were not removable by physical means for the production of antibodies at a later stage. If PMEases were left on polyacrylamide gels overnight, before BCIP staining, no activity was then detected. Carbohydrate staining also suggested that PMEase bands 1, 2 and 4 were associated with carbohydrate (Fig 5.4b).

Staining of material for 15 min in a shaken snap-cap vial with BCIP showed localization of blue colour on mucilage, sheath and surface of the hair (Fig 5.5); the blue colour was apparently due to the formation of the insoluble indigoid (Coston and Holt, 1958). Neither the surface of vegetative cells nor the cytoplasm were stained. When addition of BCIP was followed immediately by the material being placed on a slide under a coverslip, the staining reaction was quite different. The first colouration was seen within the hair by 5 min; after 15 min there was some blue colour on mucilage, sheath and hair surface, but less than when the cells were shaken in a vial. The intracellular colouration appeared initially to show a gradient from top to bottom of the filament, although it was difficult to be sure due to photosynthetic pigments in the "vegetative" cells.

Fig. 5.3 Effect of ions on cell-bound and extracellular PMEase activity in Calothrix 550. Cultures were grown for 28 d at 25°C and 60 mmol photon m^{-2} s^{-1}.

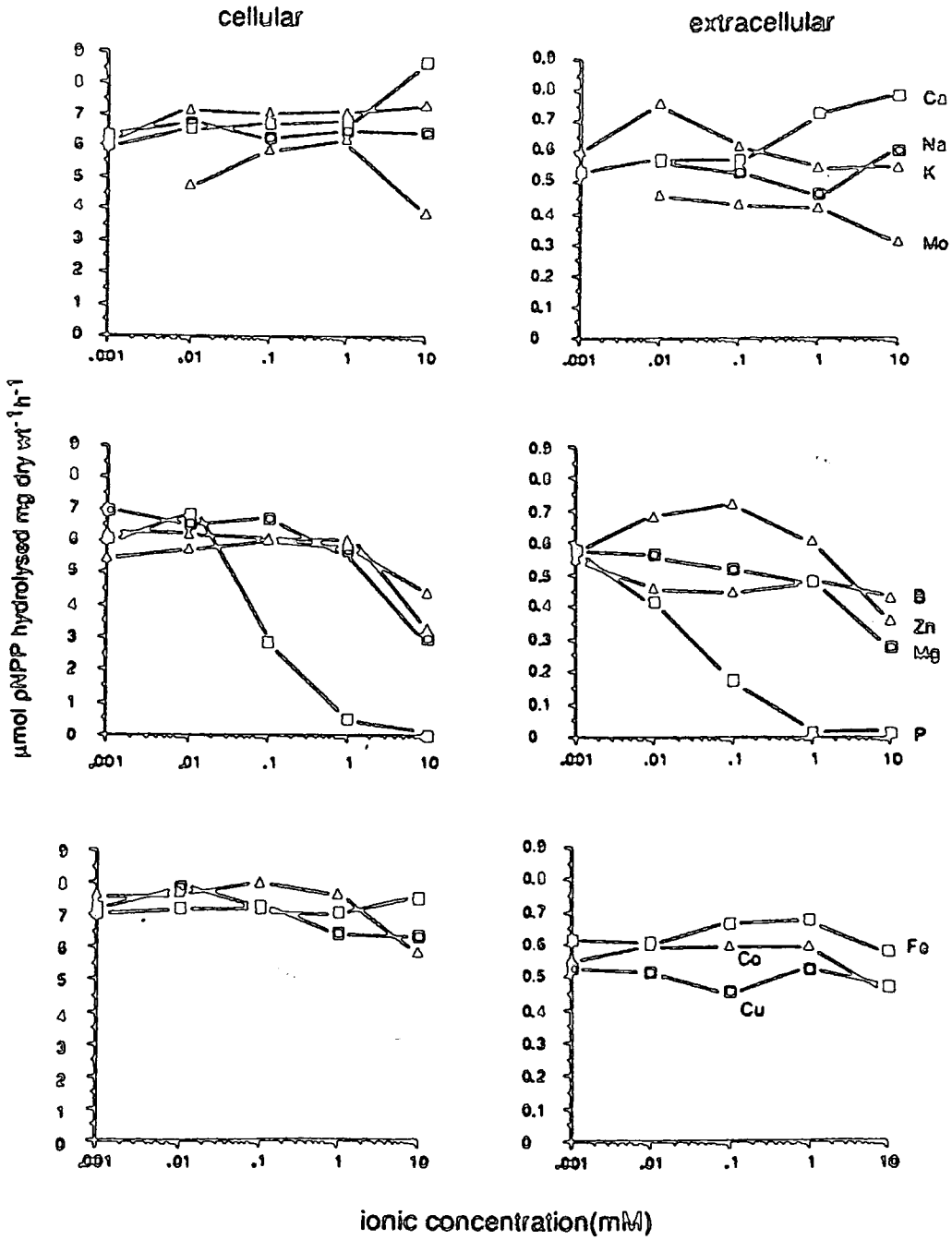


Fig. 5.4 Detection of four bands of phosphomonoesterase activity in Calothrix 550. Extracellular fraction was centrifuged at 110,000 x g for 1h, electrophoresed on a non-denaturing polyacrylamide gel and stained with BCIP. a) bands 1-4 are phosphomonoesterase activity in the pellet (track 1) and supernatant (track 2); b) representation of carbohydrate (shaded area) associated with bands 1, 2 and 4.

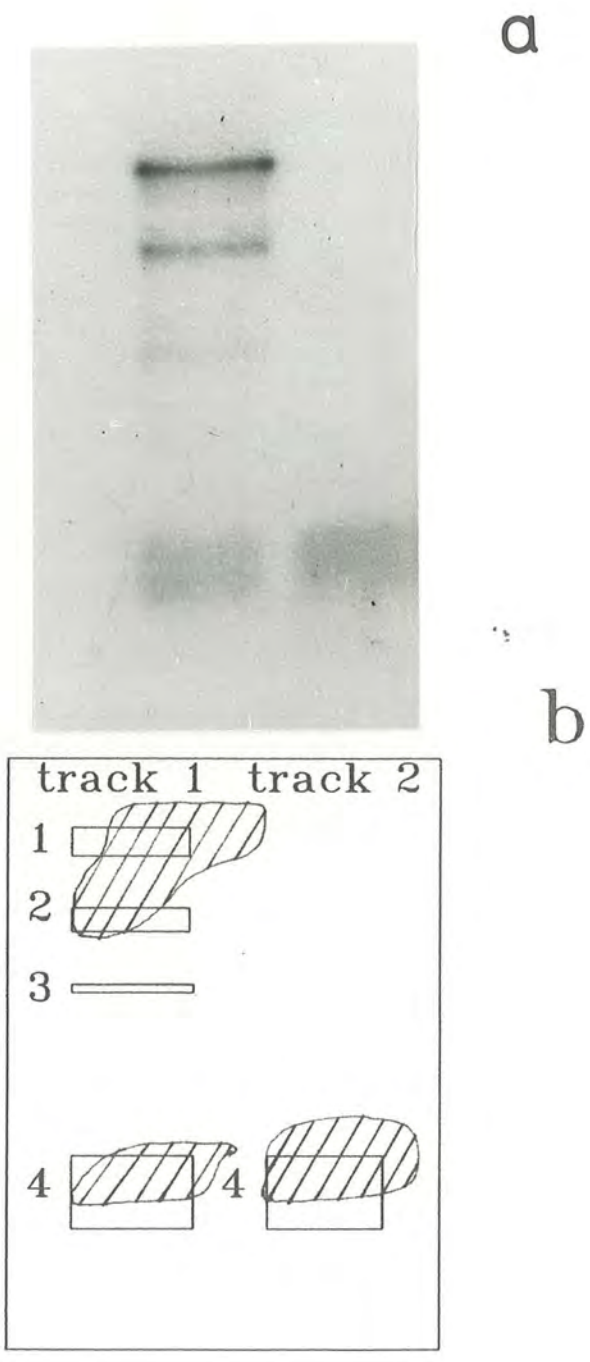
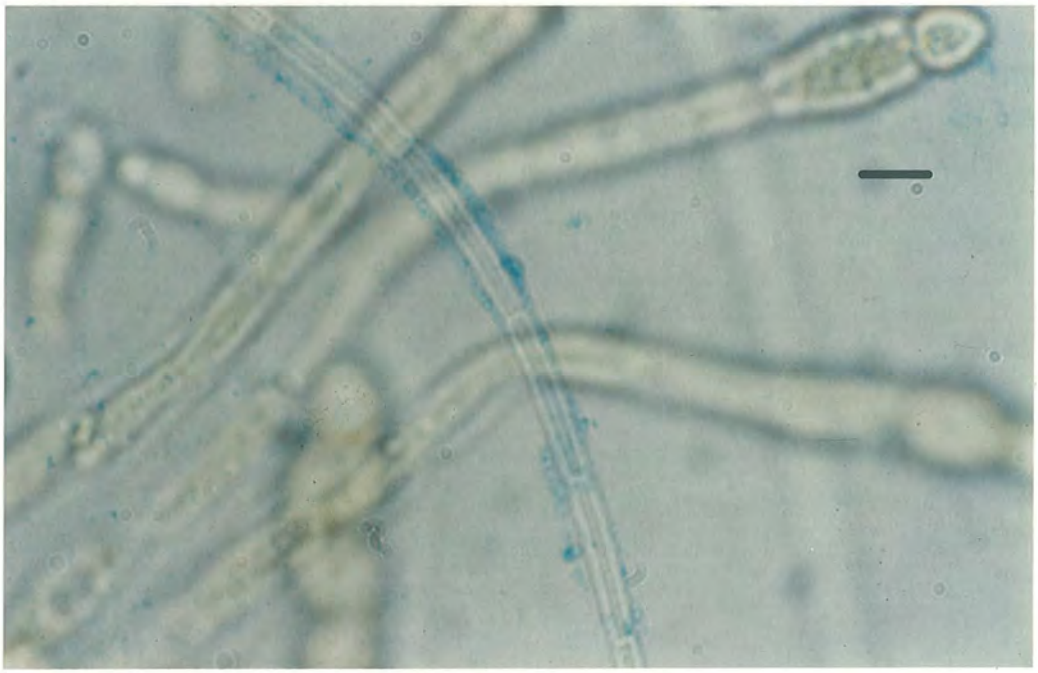


Fig. 5.5 Localization of phosphomonoesterase activity on the hairs of 16 d old Calothrix 550 using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the organic phosphorus source. Activity is localized on the hair as insoluble blue indigoid, scale bar = 10 μm.



5.4 INFLUENCE OF ρNPP CONCENTRATION ON CELL-BOUND AND EXTRACELLULAR PHOSPHOMONOESTERASE ACTIVITY IN Calothrix 550

5.41 Introduction

In the case of variation in the rate of activity with substrate concentration the reaction is represented by the Michaelis-Menten equation:

$$v = \frac{V.S}{K_m+S}$$

v is the rate at any substrate concentration, V is the maximum rate (V_{max}) attained by the reaction at high substrate concentrations and K_m, the Michaelis constant, is the substrate concentration when the rate is half the maximum attainable rate. Thus, V and K_m are experimentally determinable constants, provided the enzyme is studied under controlled conditions, and are characteristic and constant for the particular enzyme. Although it is possible to obtain the value of V_{max} and K_m directly from the graph of rate against substrate concentration it is more common to plot the reciprocal of the rate 1/v against the reciprocal of the substrate concentration. This method of expressing the results is known as the Lineweaver-Burke plot.

$$\frac{1}{S} = \frac{Km}{V.S} + \frac{1}{v}$$

5.42 Method

The dependence of cell-bound and extracellular PMEase on the concentration of ρNPP can be described on the basis of Michaelis-Menten kinetics. To determine the K_m and V_{max} a Lineweaver-Burke plot was constructed. The assays were carried out at pH 10.3 and at 32°C (3.22). Cellular material was harvested as in 5.12.

5.43 Results

Use of the Lineweaver-Burke plot, 1/v versus 1/S allowed the calculation of half saturation values and concentration of ρNPP (K_m) required to support half the maximum rates. K_m for cell-bound and extracellular PMEase was 4.34×10^{-5} M and 3.28×10^{-5} M, respectively. These values are similar to the values reported for some heterotrophic bacteria, a K_m of 1.2×10^{-5} M in E. coli PMEase (Garen and Levinthal, 1960) and 3.6×10^{-5} M in Bacillus subtilis (Thompson and MacLeod, 1974a). The similarity in K_m values suggests that cyanobacterial enzymes may have a similar structure to heterotrophic PMEases, even though the influence of ions and pH on PMEase activity was markedly different from heterotrophic bacteria.

CHAPTER 6

INFLUENCE OF SALINITY ON HAIR FORMATION AND PHOSPHATASE ACTIVITIES IN THE RIVULARIACEAE

6.1 INFLUENCE OF ORGANIC PHOSPHORUS SOURCE, TEMPERATURE, pH, IONIC COMPOSITION AND SALINITY ON HAIR FORMATION AND PHOSPHATASE ACTIVITIES IN *Calothrix* 253

6.11 Introduction

Location of PMEase activity in hairs of *Calothrix* 550 was detected using BCIP and a fraction containing >95% hairs. However, staining with naphthol AS-MX phosphate and location of activity in vegetative cells, sheath and mucilage suggests that hairs in *Calothrix* 550 may not be the only major site of phosphatase activity. It was therefore decided to work on other hair-forming strains to see if conclusive evidence could be obtained to show that hair formation and phosphatase activity were linked.

These studies were planned to investigate the properties of PMEase activity in *Calothrix viguieri* (253) and the influence of salinity on PMEase activity, in which it had been noted that increased salinity led to reduced hair formation (Mahasneh, 1988). The inhibition of hair formation by increased salinity may be used to demonstrate that phosphatase activity is directly linked to hair formation.

C. viguieri was isolated by J. Komárek (pers. comm.) from the upper part of a mangrove root in Cuba at about high tide level, and was probably subject to marked differences in salinity in nature. *C. viguieri* was obtained from the (then) Cambridge Culture Collection of Algae and Protozoa (1410/6) in 1973; a clonal axenic isolate has been maintained as Durham culture 253 (D253). The

isolate is maintained in Třeboň as C. viguieri, although listed as Calothrix sp. by CCAP.

6.12 Method

Initially Calothrix 253 was adapted to a marine medium and eventually it grew well in 30% ASP_6 medium (Provasoli et al., 1957). After two years in this medium, it showed poor growth and abnormal morphology on initial subculture to Chu 10D-N. Further subculturing in the latter medium for three months led to a return to normal morphology, but less rapid growth on subculturing to saline medium. From this point Calothrix 253 was subcultured in freshwater medium (Chu 10D-N) and saline medium (Chu 10D-N + NaCl, see below), these strains were referred to as freshwater Calothrix 253a and saline Calothrix 253c respectively in the Durham Culture Collection. Material subcultured in freshwater medium was the source of inocula for experiments unless noted otherwise.

C. viguieri was grown in batch culture at 32°C and continuous illumination at 100 μmol photon m^{-2} s^{-1}, with the inoculum at c. 10 mg l^{-1}. Most experiments were made in either Chu 10D-N medium or a version with added NaCl. Experiments with added NaCl used 5.6 g l^{-1} (= 90 mM NaCl), giving approximately 20% of the Na concentration in seawater of salinity 35 °/oo (Head, 1983). This medium is referred to as Chu 10D-N + NaCl.

The ability of the alga to grow in a range of P sources was tested in freshwater and saline medium, material was harvested after 16 d (2.441) and assayed for cell-bound and extracellular PMEase (2.45) and PDEase (2.442) activities.

The influence of temperature on cell-bound PMEase was tested (5.12). The influence of pH (2.45) on activity was tested using a range of buffers (Table 6.1), with two buffers tested at each pH value. Between pH 10.6-12.6

different amounts of NaOH were added to either glycine or AMeP. The pH was monitored during assays using a Cardy compact pH meter (2.41).

The effects of Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Zn^{++} and phosphate on cell-bound PMEase activity were determined using 0.001, 0.01, 0.1, 1 and 10 mM concentrations in the assay mixture (5.31). Localization of PMEase activities in freshwater and saline versions were tested using naphthol AS-MX phosphate as the organic P source (2.731).

To test the effect of transferring cultures from saline to freshwater medium cultures were grown in Chu 10D-N + NaCl for 16 d and transferred to freshwater medium without added P. Material was assayed for cell-bound PMEase activity (2.45) and preserved for microscopical analysis at hourly intervals for 24 h. In order to test whether the influence of NaCl on hair formation and phosphatase activity was an osmotic effect, cultures were grown in the presence of mannitol or sorbitol at concentrations up to 135 mM. Polymixin B sulphate at 1, 5, 10, 15 and 20 units/ml was added to cultures transferred from saline medium to freshwater medium without added P to investigate its effect on hair formation.

Experiments were carried out to determine whether the response to an increase in NaCl could be modified by changing the concentrations of Ca in the saline medium. Saline adapted material was grown to P-limitation (16 d) in Chu 10D-N medium (normal concentration 0.234 mM Ca) with 67.5 mM NaCl and Ca at 0.234, 1, 5, 10 and 20 mM. 67.5 mM NaCl was chosen as this was the concentration where hair formation just began.

Table 6.1 Buffers used in conjunction with NaOH for testing the influence of pH on cell-bound PMEase and PDEase activity in Calothrix 253. The buffer which led to the higher activity and was used for data in Fig. 6.2 is indicated here, unless activity was below the detection limit (< 0.02 µmol ρNP hydrolyzed mg dry wt^{-1} h^{-1}).

Abbreviations (Table 5.1)

pH	buffer A	buffer B	NaOH (mM - final conc.)	buffer used
7.0	DMG	HEPES		DMG
8.0	TES	HEPES		TES
9.0	AMeP	glycine		AMeP
10.0	AMeP	glycine		AMeP
10.3	AMeP	glycine		AMeP
10.6	AMeP	glycine	8	AMeP
10.8	AMeP	glycine	13	AMeP
11.0	AMeP	glycine	17	AMeP
11.2	AMeP	glycine	19	AMeP
11.4	AMeP	glycine	20	AMeP
11.6	AMeP	glycine	22	AMeP
11.8	AMeP	glycine	24	AMeP
12.0	AMeP	glycine	26	AMeP
12.2	AMeP	glycine	34	AMeP
12.4	AMeP	glycine	52	AMeP
12.6	AMeP	glycine	78	AMeP

6.13 Results

The ability of the alga to grow in a range of P sources was tested in freshwater and saline medium. The yield was in all cases either equal or lower in saline medium (Table 6.2). There was a seven-fold increase in yield compared with medium lacking any P source (control). In freshwater medium all organic P sources tested led to a higher yield than the control, but in saline medium ATP, bis-ρNPP and DNA led to yields below the control. Use of these three organic P sources led to the filaments appearing very unhealthy at the time of harvest, with the effect being more marked in the case of saline medium; this contrasts to the situation with the control where the filaments still had a more or less normal morphology.

Cell-bound PMEase activity was absent in P-rich cultures, but was induced in P-limited cultures. However the difference between cell-bound PMEase activities of alga grown in freshwater and saline media (Table 6.2) was much greater than that of their yields. Cell-bound PDEase activity was inducible in freshwater medium, but was not detected in saline medium. There were marked differences in the ratio of (cell-bound) PDEase to PMEase (Table 6.2), with the highest values (1 : 10, expressed as ρNP released) for cultures grown in ρNPP or bis-ρNPP. In spite of the absence of detectable cell-bound PDEase activity in cultures grown with DNA as P source, the yield in freshwater medium was twice that of the control. Extracellular PMEase and PDEase activities were not detected in any experiment.

When the same concentration of NaCl as that used in saline growth medium was included for assays of cultures grown in freshwater medium, mean decreases of 33% and 19% for cell-bound PMEase and PDEase activities, respectively were recorded. These differences are much less than those for the activities

subsequent to growth in the two different media, showing that the reduced activity is not simply due to inhibitory effects of NaCl during this assay.

Morphological observations on cultures grown to P limitation in freshwater and saline media showed that the trichomes in the former ended in long hairs. Azo-dye staining in freshwater and saline versions transferred to freshwater medium (>12 h) for cell-bound PMEase activity showed that there was a sharp transition between the hair with activity and the remainder of the trichome in both (Fig. 6.1). There was no detectable staining of trichomes, grown to P-limitation, in saline medium grown to P limitation.

PMEase activity of cultures grown in freshwater medium was undetectable below pH 7.0 and maximal at pH 12.2 (Fig. 6.2b). Maximum activity was at 50°C and above 80°C activity was undetectable (Fig. 6.2a); these values may be compared with growth on a temperature plate, where the optimum was 32°C and minimum and maximum temperatures for survival in culture were 13°C and 39°C, respectively. The influence of Na^+, K^+, Ca^{++}, Mg^{++}, Zn^{++} and phosphate was tested over the concentration range 0 - 10 mM (Fig. 6.3a,b). The most obvious effects were a marked rise in activity with increased Ca and decreased activity at higher concentrations of Mg and phosphate; there was no detectable PMEase activity in the absence of Ca. The decrease in activity due to Na was negligible over this range although 90 mM NaCl led to a 33% reduction in activity (see above).

Transfer of cultures from saline to freshwater medium led to the development within 24 h of a long hair at the end of 90% of the trichomes (Fig. 6.4d). The morphological change was especially well synchronized when the inoculum was taken from a culture fully adapted to growth in saline medium. The formation of hairs and a rise in PMEase activity shown by hydrolysis of pNPP commenced at the same time (Fig. 6.4).

The addition of P to the P-limited cultures led to release of hormogonia in both saline and freshwater media, but whereas in the former the hormogonia

were differentiated from the terminal part of the trichome, in the latter they formed below the hairs, with the hairs being shed.

The response of material with long hairs developed in freshwater medium to transfer to saline medium was for the hairs to be shed and to float to the surface. About half the hairs were shed within 24 h and most within three days; no separation disc was formed during the loss of the hair. Increase of NaCl also led to the development of intrathylakoidal vacuoles in many vegetative cells, especially towards the apical end of the trichome and typically with one vacuole adjacent to the cross-wall at the apical end of a cell.

There was an indication that Ca may modify the influence of NaCl on hair formation. After 16 d in saline medium hairs were 20 - 30 μm long and %hairiness was 2-5% in 0.234 and 1 mM Ca. However with higher Ca concentrations (5-20 mM) %hairiness increased to 80% and hair length was between 80-150 μm, but the cultures subsequently became brown and many trichomes lyzed.

There was no detectable effect on hair formation, PMEase and PDEase activities, or localization of PMEase activity, shown by azo dye staining with varying concentrations of mannitol or sorbitol.

There was no effect on hair formation or PMEase activity after 24 h when cultures were transferred from saline medium to freshwater medium with 1, 5, 10 or 15 units ml^{-1} of polymixin B sulphate. However, with 20 units ml^{-1} of polymixin B sulphate, hair formation and PMEase activity was inhibited after 24 h. However at 48 h vegetative cells showed signs of bleaching.

Fig. 6.1 Tapered part of the trichome in Calothrix 253, showing sharp transition between hair cells (h) stained for PMEase activity and vegetative cells lacking activity. Staining was carried out with naphthol AS-MX phosphate as the organic P source and Fast Blue RR diazonium salt as the coupling agent. (Similar results obtained when hairs were formed as a direct response to P limitation or due to transfer from saline to freshwater medium). Scale bar = 10 μm.

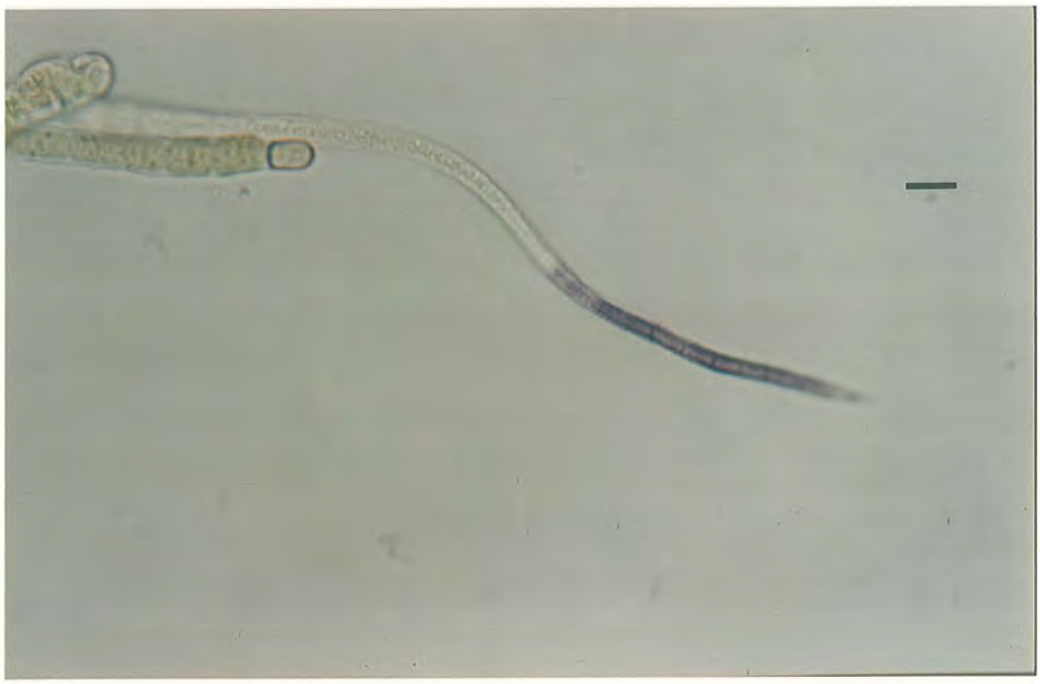
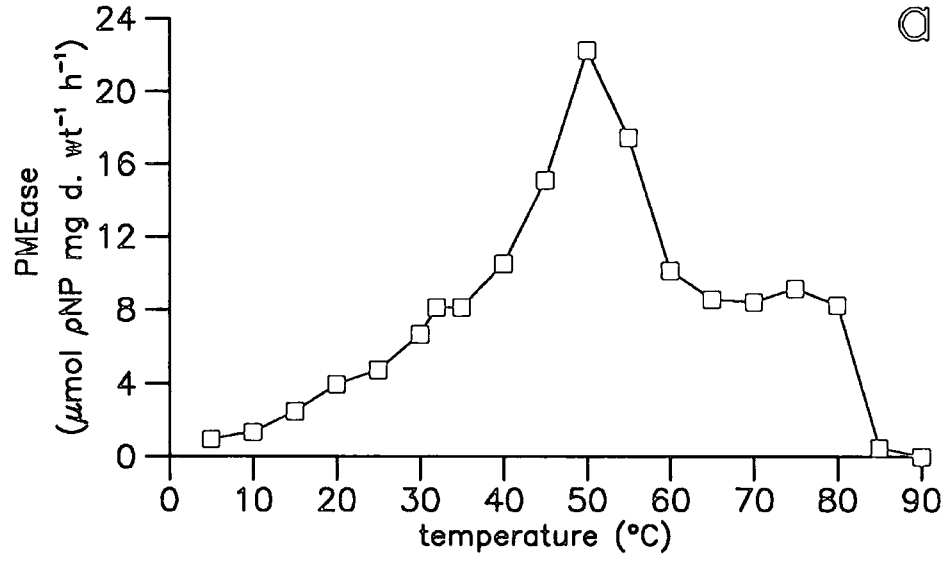


Fig. 6.2 Influence of temperature (a) and pH (b) on cell-bound PMEase activity in Calothrix 253. Cultures were grown for 16 d at 32°C and 100 μmol photon m^{-2} s^{-1}.



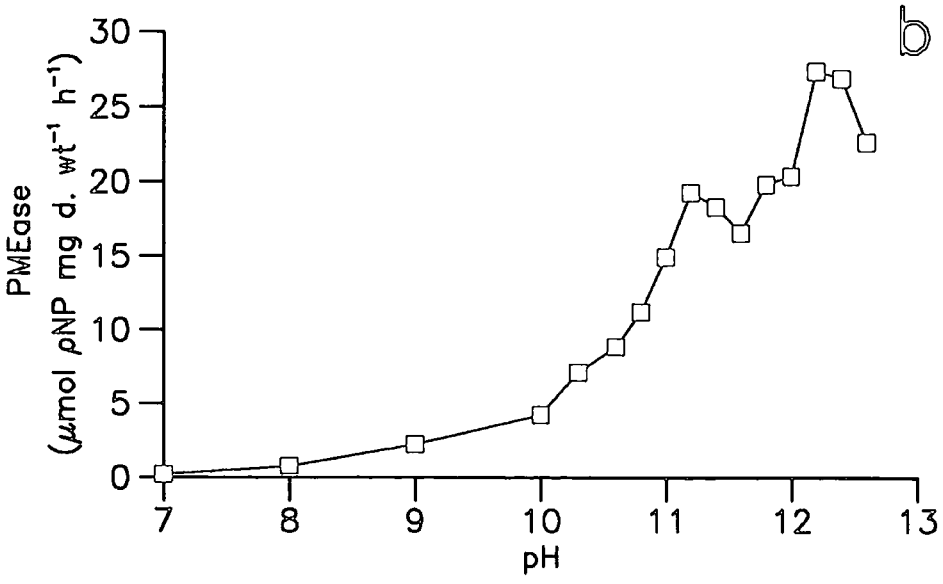


Fig. 6.3 Influence of ionic composition on cell-bound PMEase activity in Calothrix 253. Cultures were grown for 16 d at 32°C and 100 μmol photon m^{-2} s^{-1}.

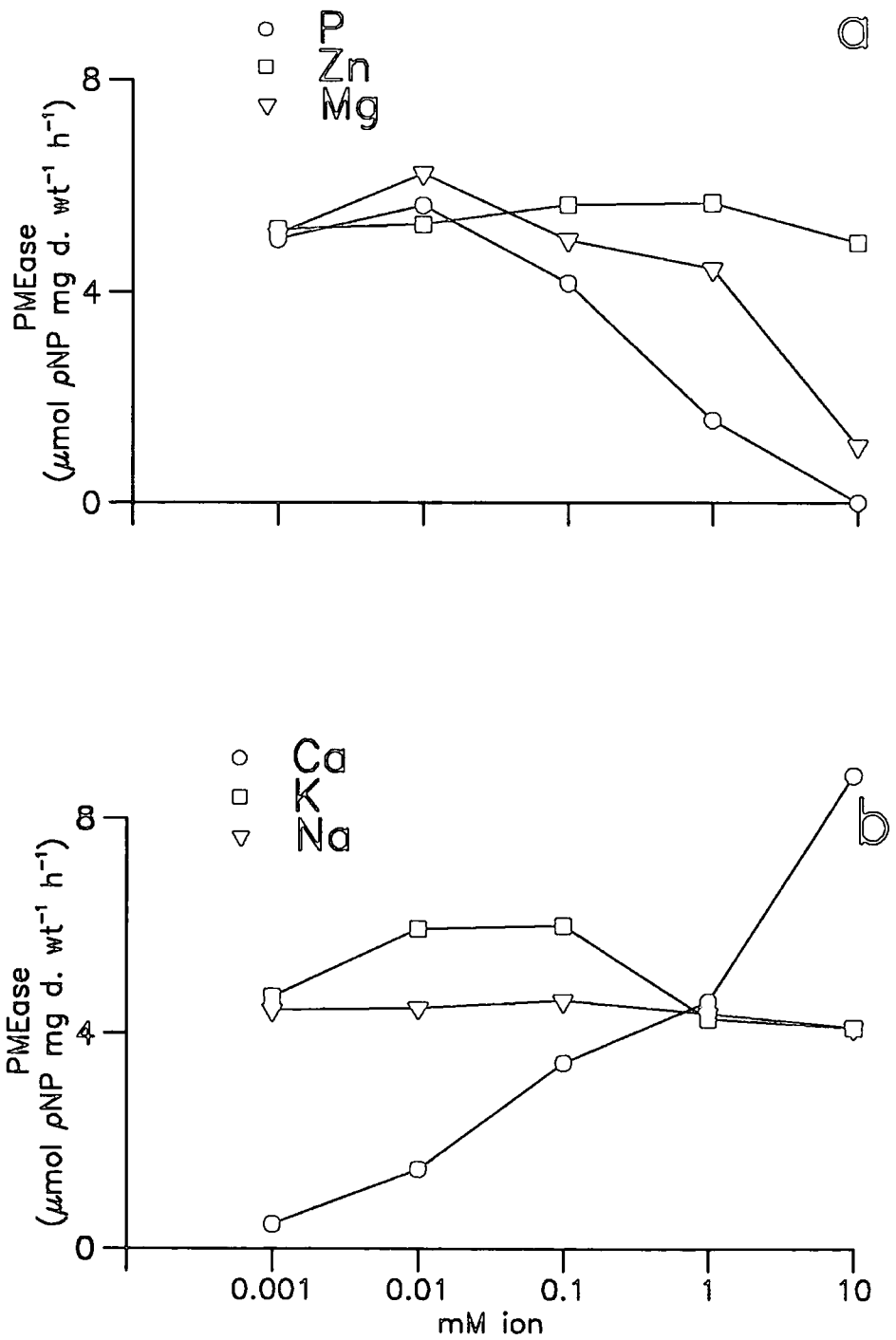


Fig. 6.4 Time course of changes in morphology and cell-bound PMEase activity of P limited Calothrix 253 following transfer from saline to freshwater medium: a) PMEase activity; b) trichome length; c) hair length; d) % trichomes with hairs.

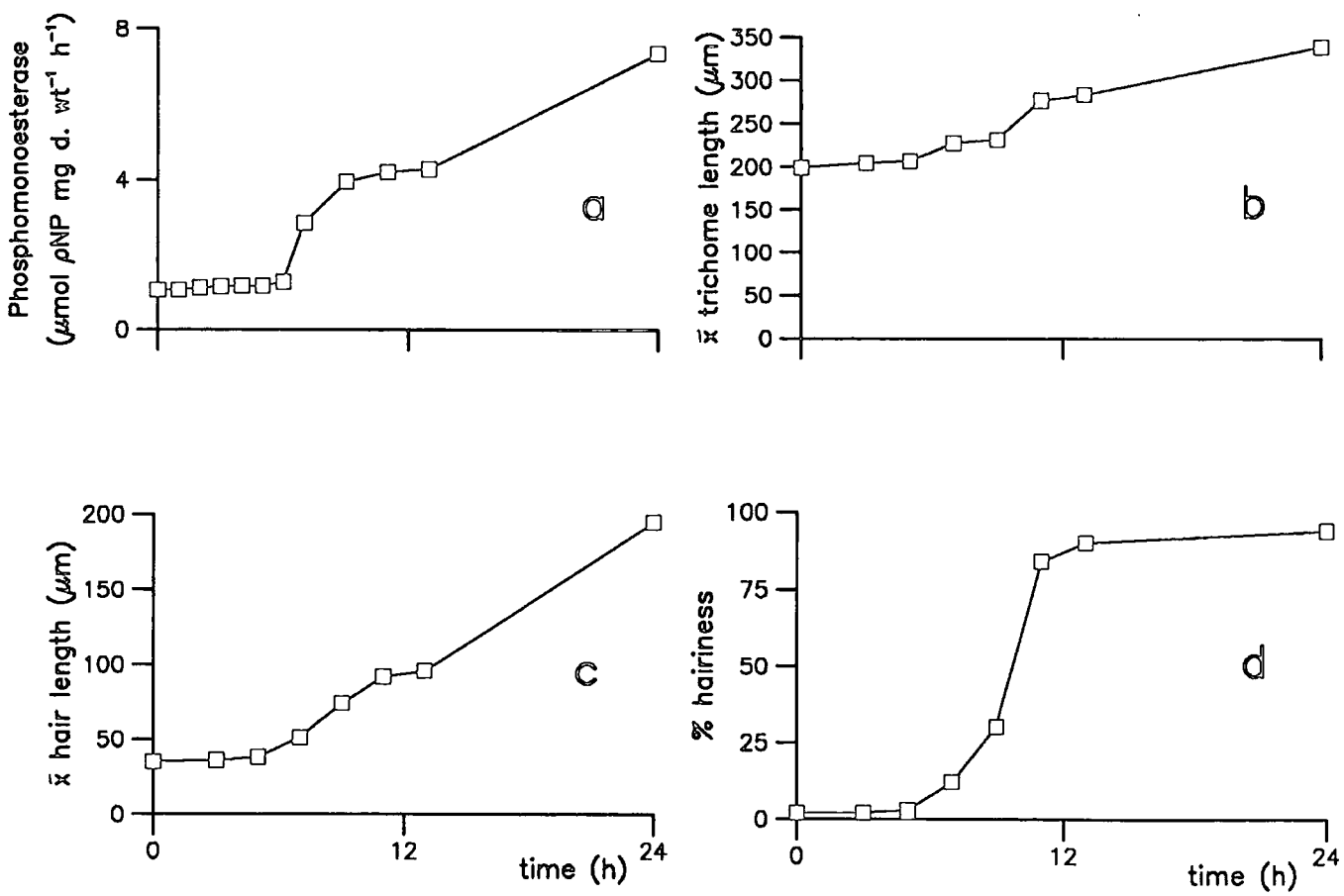


Table 6.2. Influence of salinity on yield and cell-bound PMEase and PDEase activities of *Calothrix* 253 after batch culture with various P sources (all 1 mg l^{-1} P). Inoculum 10 mg l^{-1} d. wt; cultures grown for 16 d at 32°C and 100 µmol photon m^{-2} s^{-1} PAR; n = 4.

	yield (mg l^{-1})		PMEase (µmol ρNP mg d. wt^{-1} h^{-1})		PDEase (µmol ρNP mg d. wt^{-1} h^{-1})	
medium	- NaCl	+ NaCl	- NaCl	+ NaCl	- NaCl	+ NaCl
no added P	74.7 ± 3.9	65.0 ± 8.8				
P_i	431.4 ± 21.6	393.7 ± 28.0	5.16 ± 0.38	0.34 ± 0.03	0.06 ± 0.005	< 0.02
ρNPP	216.3 ± 26.1	142.0 ± 12.5	2.42 ± 0.15	0.16 ± 0.01	0.26 ± 0.02	< 0.02
MNP	170.7 ± 9.7	88.0 ± 2.8	0.73 ± 0.11	< 0.02	0.06 ± 0.003	< 0.02
β-glyceroP	209.7 ± 15.2	193.0 ± 12.8	1.70 ± 0.08	0.20 ± 0.01	0.06 ± 0.008	< 0.02
ATP	139.7 ± 2.0	41.4 ± 4.9	0.44 ± 0.09	< 0.02	< 0.02	< 0.02
bis-ρNPP	131.9 ± 7.2	37.0 ± 4.2	5.37 ± 0.58	< 0.02	0.49 ± 0.08	< 0.02
DNA	143.2 ± 4.9	50.2 ± 3.8	0.14 ± 0.01	< 0.02	< 0.02	< 0.02
phytic acid	193.7 ± 13.3	191.0 ± 12.1	6.51 ± 0.90	0.18 ± 0.01	0.07 ± 0.007	< 0.02

6.2 INFLUENCE OF SALINITY ON 8 HAIR-FORMING RIVULARIACEAE

6.21 Introduction

Hair formation in Calothrix 253 (6.1) was inhibited by NaCl (>67.5 mM-), which resulted in indication of the association between hair cells and PMEase activity in Calothrix 253. A further eight hair-forming Rivulariaceae (Table 2.1) were chosen to see if the effect of NaCl was repeatable in other strains. If any strains showed a similar response, further work on the effect of pH on cell-bound PMEase activity would be carried out. The effect of pH was chosen, as this was the most characteristic feature of cell-bound PMEase activity in Calothrix 253.

6.22 Method

Eight strains were chosen: 1. Calothrix 184, 2. Calothrix 251, 3. Calothrix 266, 4. Calothrix 550, 5. Calothrix 572, 6. Calothrix 690, 7. Dichothrix 808, 8. Dichothrix 809.

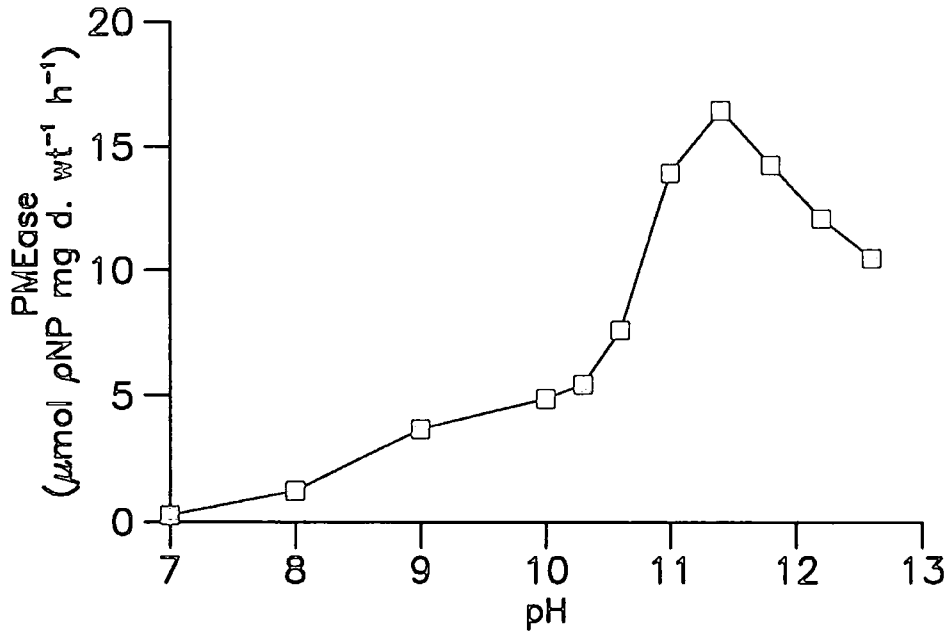
These strains were grown for 16 d in Chu 10D-N + NaCl at 32°C and 100 µmol photon m^{-2} s^{-1} (2.562). The concentrations of NaCl used were 45, 67.5, 90, 112.5 and 135 mM. After 16 d the cultures were examined for changes in hair formation (2.72). The effect of pH between pH 7.0 - 12.6 on cell-bound PMEase activity was investigated as in 6.12 and duplicate buffers were used at each pH value (Table 6.1).

6.23 Results

There was no effect on hair formation in seven of the eight strains tested. However, in Calothrix 690 NaCl had a marked effect. There was a gradual decrease in hair formation above 45 mM NaCl and no hairs formed at 135 mM NaCl. The decrease in hair formation in Calothrix 690 was not as sharp as in Calothrix 253.

The effect of pH on cell-bound PMEase activity in Calothrix 690 was very similar to Calothrix 253 with a high pH optimum of 11.8 (Fig. 6.5). Previous to this work the highest PMEase pH optima reported were between pH 9.0 - 10.4. It is interesting to note that the two major similarities between Calothrix 253 and 690 are the effect of NaCl on hair formation and the high pH optima of cell-bound PMEase activity.

Fig. 6.5 Influence of pH (7.0-13.0) on cell-bound PMEase activity in Calothrix 690. Culture was grown for 16 d at 32°C and 100 μmol photon m^{-2} s^{-1}.



6.3 EFFECT OF REDUCING NaCl IN CHU 10D-N ON HAIR FORMATION IN 12 NON HAIR-FORMING RIVULARIACEAE

6.31 Introduction

Hair formation in Calothrix 253 and 690 was inhibited by increasing the NaCl concentration above 67.5 mM in Chu 10D-N. Of the 30 Rivulariaceae strains tested in Chapter 3, 21 did not form hairs when P-deficient (3.3), although hair formation was similar in some of the field isolates. Hair formation in these strains may be inhibited by the relatively high levels of Na in Chu 10D-N in comparison to the levels of Na in the field Therefore, 12 selected strains were grown in medium with reduced sodium until P-deficient, and observed for hair formation or changes in trichome structure.

6.32 Method

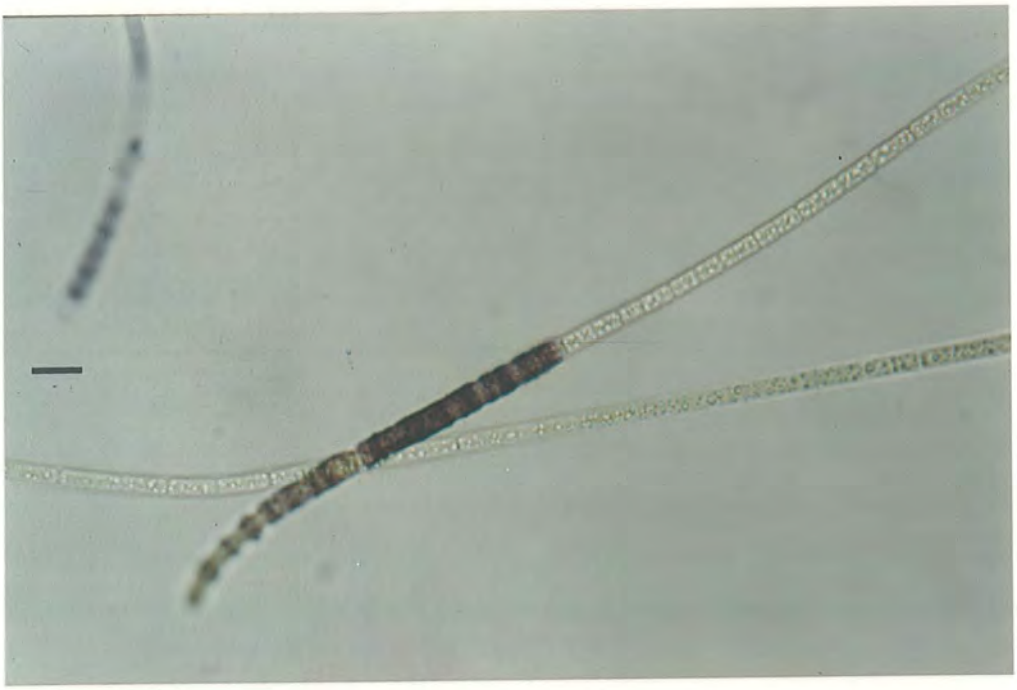
In Chu 10D-N (Table 2.4 and 2.6) HEPES was reduced to 0.06g l^{-1} and buffered at pH 7.6 with 1 M KOH. K increased from 0.057 mM to 0.511 mM, P was reduced from 0.032 mM to 0.025 mM and 0.23 ml of the $NaHCO_3$ stock was added to 1 litre of medium. The final concentration of sodium was reduced from 1.67 mM to 0.022 mM. Cultures were grown for 16 d in the growth room (2.561) at 32°C and 60 µmol photon m^{-2} s^{-1} in a 14 h light and 10 h dark cycle. This was the closest comparison to field conditions. After day 16, cultures were compared against controls for any changes in trichome morphology (2.772). Cell-bound PMEase activity at pH 10.3 (2.441) and localization of activity using azo-dye (2.731) were determined in strains with a marked response to a reduction in NaCl. The non hair-forming strains were Calothrix 603, 624, 730, 764, 794, 795, 796 and 802 and Gloeotrichia 281, 613, 626 and 743. Calothrix 550 was used as a control.

6.33 Results

There were no marked changes in trichome morphology except in Calothrix 764 and Gloeotrichia 281 and 613. In these strains there were marked increases in trichome length. The cultures appeared healthy, although vacuolation was noted in the tips of the longer trichomes. No cell-bound PMEase activity was localized using azo dye in Gloeotrichia 281 and 613, which were similar to the results previously obtained for these strains (Table 3.13), suggesting that the strains were unable to hydrolyze naphthol AS-MX phosphate. However, in Calothrix 764 activity was localized in the tips of the trichomes, where extension and vacuolation had occurred (Fig. 6.6). A completely different pattern of localization occurred in the control. The entire filament in mature trichomes was stained and no specific localizations in the apices were noted (Table 3.13, 3.3).

Assays of cell-bound PMEase activity in Gloeotrichia 281 and 613 and Calothrix 764, using ρNPP, showed no marked differences to the controls. These results suggest that although trichome morphology had changed markedly, the type and level of PMEase activity had possibly been redistributed. The reduction in Na had no effect on hair formation in Calothrix 550.

Fig. 6.6 Cell-bound PMEase activity localized in the tapered part of the trichome in Calothrix 764. Culture was grown in reduced sodium for 16 d. Staining was carried out with naphthol AS-MX phosphate as the organic P source and Fast Blue RR diazonium salt as the coupling agent. Scale bar = 10 μm.



CHAPTER 7

PO_4 UPTAKE IN SALINE AND FRESHWATER VERSIONS OF *Calothrix* 253 AND IN *Calothrix* 550

7.1 INTRODUCTION

In Chapters 5 and 6 phosphatase activity and its relationship with hair formation and trichome structure in saline *Calothrix* 253 and freshwater *Calothrix* 253, *Calothrix* 550 and *Calothrix* 690 were studied. Previous experiments suggested that hair formation, phosphatase activity and effective hydrolysis of external high concentrations of organic P in the environment are all linked. In order to utilize effectively P_i released from the hydrolyzed organic P sources, these strains must also possess a system for P_i uptake at high external concentrations. An uptake system for high NH_4-N concentrations was proposed by DeBoer and Whoriskey for *Ceramium rubrum* (1983, 1.4) and this uptake system was located in the hairs of the alga.

As hairs are sites of organic P hydrolysis and thus the site for release of P_i, it is likely that they are also a site for P_i uptake. It was therefore decided to conduct PO_4 uptake experiments in *Calothrix* 253 and 550, to see if there was an association between hair formation and PO_4 uptake in the Rivulariaceae. The K_m and V_{max} of P_i uptake, the influence of light and dark, metabolic inhibitors, pH and ions on P_i uptake were examined.

Use of saline and freshwater versions of *Calothrix* 253 provided a means whereby direct comparison could be made on PO_4 uptake (as in phosphatase studies, 6.1) in a species with and without hairs and in the same P status. *Calothrix* 550, a marked hair-forming strain, was used to compare V_{max} and K_m values for P_i uptake reported in other cyanobacteria (1.72).

7.2 METHOD

Algae were grown in 1-litre flasks, shaken twice a day to prevent CO_2 limitation, for 16 d at 32°C and 60 μmol photon m^{-2} s^{-1} and harvested as before

(2.441). They were then resuspended in Chu 10D-N,-P (2.531), buffered at pH 7.6 with 0.25 mM HEPES (2.54), lightly homogenised through a 50-ml syringe and divided into 20-ml aliquots in 100-ml flasks. The flasks were incubated in a growth room at 32°C and 60 µmol photon m^{-2} s^{-1} (2.561) on an orbital shaker. 4 x 20 ml aliquots were used for dry wt (2.57) and cellular P (2.61) determination. All flasks were pre-incubated for 1 h before experiments on P_i uptake were carried out. P_i (pre-incubated to 32°C) was added at different concentrations and experiments ran between 4 and 20 min. Reactions were terminated by passing 10-ml aliquots of homogenate through GF/F filters. Soluble reactive phosphorus (SRP) remaining in the filtrate was determined (2.61) and P_i uptake was recorded as µmol P mg dry wt^{-1} h^{-1}.

The influence of light, dark and 20 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was tested on P_i uptake. DCMU is a direct inhibitor of the Hill reaction and an indirect inhibitor of photosystem I. The effects of these variables on P_i uptake were determined using KH_2PO_4 between 0-60 µM. Flasks were incubated in the dark (2.42) for 1 h. DCMU was added 10 min prior to the addition of P_i.

The influence of pH, between pH 4-11, was tested at two different concentrations of PO_4. Duplicate buffers were used at each pH value (Table 2.3), the buffer which led to the higher P_i uptake value (Table 7.1) was used in Figs 7.1 and 7.2. The effect of 0.01 mM, 0.1 mM, 1 mM and 10 mM Ca^{++} and Mg^{++} (5.32) on P_i uptake was determined using 15 µM PO_4. K_m and V_{max} values were determined from Lineweaver-Burke plots (5.4).

Table 7.1 Buffers, which led to higher P uptake values, used for experiments in Figs 7.1c and 7.2c to determine the influence of pH on PO_4 uptake in a freshwater version of Calothrix 253 and in Calothrix 550.

pH	f/w 253 PO_4 uptake buffer	550 PO_4 uptake buffer
4	-	-
5	-	DMG
6	DMG	DMG
7	HEPES	HEPES
7.6	HEPES	HEPES
8	HEPES	TES
9	AMeP	glycine
10	AMeP	glycine
11	-	-

7.3 RESULTS

Highest V_{max} values were in Calothrix 550 and the lowest values were in saline Calothrix 253 (Table 7.2). Dark and 20 µM DCMU reduced K_m and V_{max} values in freshwater Calothrix 253 and Calothrix 550 (Figs 7.1a,b and 7.2a,b). P_i uptake was reduced by 60% at concentrations below 20 µM PO_4 in freshwater Calothrix 253 and Calothrix 550 . However, at higher concentrations (20 µM and above) there was no observable inhibition of P_i uptake in the dark or with DCMU.

The highest V_{max} value in freshwater Calothrix 253 was 0.852 µmol P mg^{-1} h^{-1} and in saline Calothrix 253, at a similar state of P-deficiency (Table 7.2), the V_{max} was 0.221 µmol P mg^{-1} h^{-1}. The ratio of K_m and V_{max} in freshwater and saline Calothrix 253 is also lower in saline Calothrix 253. It was suggested by Healey (1982) that the ratio of K_m to V_{max} was a better indication of P_i uptake in algae. Low P_i uptake, K_m (high affinity) and V_{max} (low velocity)

values in saline Calothrix 253, non-hairy (P-deficient), suggest that P_i uptake in this version was effective at low P_i concentrations. P_i uptake values in hairy freshwater Calothrix 253 were higher at both high and low P_i concentrations. This suggests that different P_i uptake systems may exist in the two versions of Calothrix 253.

The absence of light and 20 μM DCMU reduced uptake values between 0-20 μM PO_4, but had no effect at higher PO_4 concentrations in freshwater Calothrix 253 and 550.

The influence of pH and ions on P_i uptake on saline Calothrix 253 was not determined as P_i uptake values were very low at standard conditions (Fig. 7.2a). The influence of pH was similar at 15 and 50 μM PO_4 in freshwater Calothrix 253 and 550, except that greater P_i uptake occurred at 50 μM PO_4. There was no P_i uptake at pH 4 and 11 in Calothrix 550 and optimal P_i uptake was at pH 8 at 15 and 50 μM PO_4 (Fig. 7.1c). In freshwater Calothrix 253 optimal P_i uptake was at pH 9, for both 15 and 50 μM PO_4, and there was no uptake at pH 4, 5, 10 and 11 (Fig. 7.2c). At pH 9 uptake had increased by 100% in freshwater Calothrix 253 in comparison to pH 7.6 (Fig. 7.2c).

Mg^{++} concentrations above 0.1 mM inhibited P_i uptake in Calothrix 550 (Fig. 7.1d), Ca^{++} had no effect on uptake. In freshwater Calothrix 253, Ca^{++} had a slight stimulatory effect and Mg^{++} had no observable effect on uptake (Fig. 7.2d).

Table 7.2 Influence of light, dark, 20 μM DCMU on K_m, V_{max} and the ratio of K_m and V_{max} in Calothrix 550 and in saline and freshwater versions of Calothrix 253.

	cellular P (% dry wt)	K_m	V_{max}	K_m : V_{max}
light f/w 253	0.42	14.836	0.852	17.413
light saline 253	0.48	1.353	0.221	6.122
dark f/w 253	0.42	7.870	0.544	14.466
DCMU f/w 253	0.42	5.586	0.642	8.700
light 550	0.37	12.930	1.757	7.359
dark 550	0.37	4.421	1.438	3.074
DCMU 550	0.37	5.227	1.597	3.273

Fig. 7.1 Effect of environmental variables on PO_4 uptake in P-deficient Calothrix 550: a) light and dark; b) 20 μM DCMU; c) pH 4-11; d) 0.01-10 mM Ca^{++} and Mg^{++}.

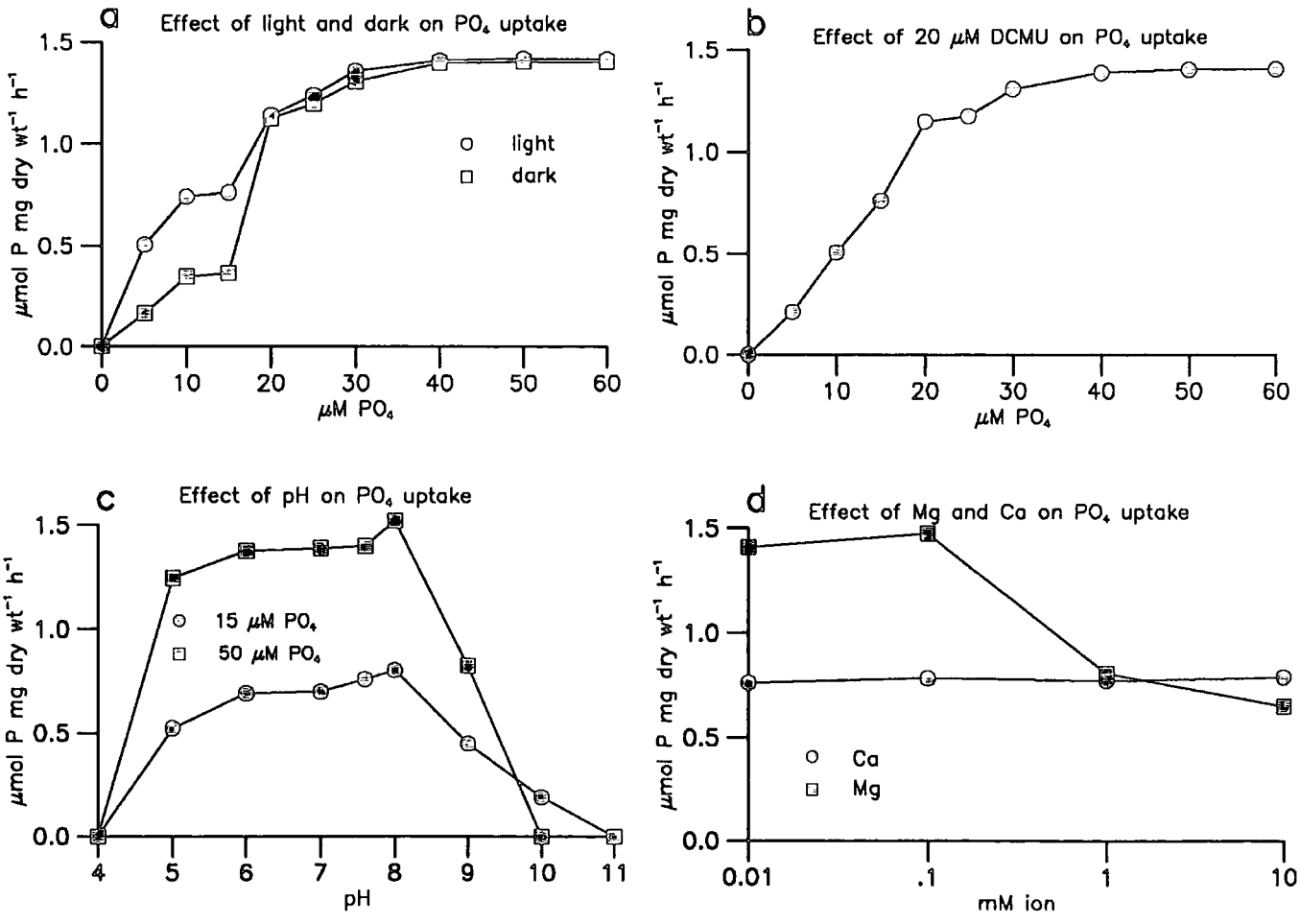
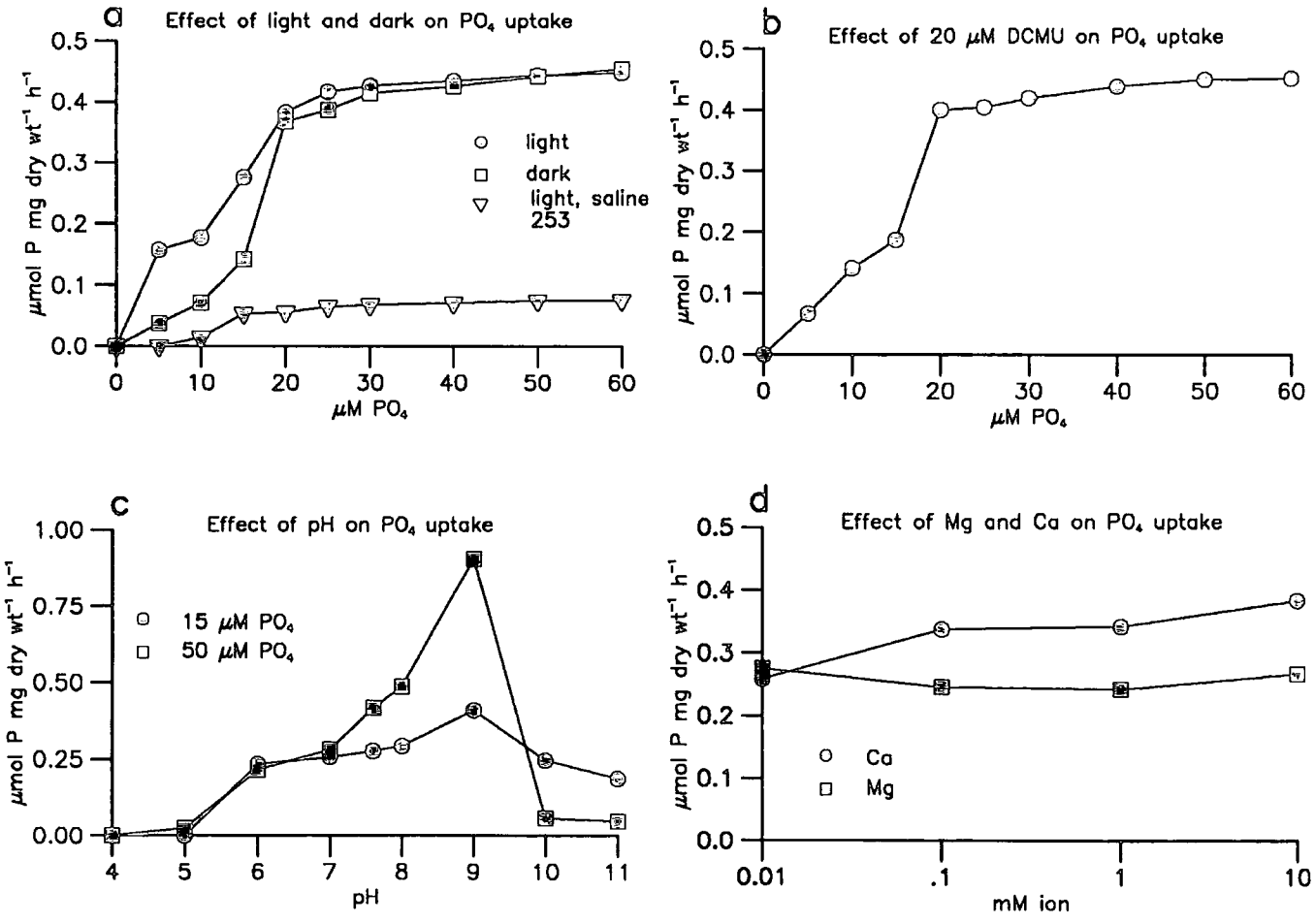


Fig. 7.2 Effect of environmental variables on PO_4 uptake in P-deficient freshwater and saline versions of Calothrix 253: a) light and dark; b) 20 μM DCMU; c) pH 4-11; d) 0.01-10 mM Ca^{++} and Mg^{++}.



CHAPTER 8

LOCALIZATION OF PHOSPHOMONOESTERASE ACTIVITY IN HAIR-FORMING EUKARYOTIC ALGAE

8.1 INTRODUCTION

Localization of cell-bound PMEase activity in hair-forming Rivulariaceae, using the azo dye naphthol AS-MX phosphate (2.732), showed that hair cells were the major site of cell-bound PMEase activity (3.3, Fig 6.1). Previous research (Whitton, 1988) showed that hairs in eukaryotic algae were also possible major sites of phosphatase activity, although the staining techniques used were not very specific.

Naphthol AS-MX phosphate had been developed as an efficient substrate to localize cell-bound PMEase activity in cyanobacterial strains (2.732). However this technique had not been used on eukaryotic algae. Therefore, it was decided to see if localization of PMEase activity on hairs, using naphthol AS-MX phosphate, also occurred in eukaryotic algae.

Three hair-forming eukaryotic algae Draparnaldia sp., Batrachospermum sp. and Lemanea sp. were obtained from calcareous field sites with high concentrations of organic P. Draparnaldia sp. and Batrachospermum sp. originated from a stream at Middleton Quarry, Teesdale, NE England and Lemanea sp. originated at a ford on the River Wear, Stanhope, NE England. The pH optimum of cell-bound PMEase activity in Batrachospermum sp. was also tested to compare with the optima in hair-forming Rivulariaceae.

8.2 METHOD

Field material was collected in source water in glass snap-cap vials and stored at ca. 4°C. Material was used immediately after returning from the field sites. Material was washed several times in assay medium (2.532) to remove debris and stained for cell-bound PMEase activity using naphthol AS-MX

phosphate (2.732). The influence of pH on activity of cell-bound PMEase activity in Batrachospermum sp. was tested between pH 3-11 in universal bottles (2.431, 2.536) at 32°C and 100 µmol photon m^{-2} s^{-1}. Duplicate buffers were used at each pH value (Table 2.3), the buffer giving the higher activity was used in Fig. 8.2.

8.3 RESULTS

Distinct localization of PMEase activity was detected only in the hair cells in Draparnaldia sp. and Batrachospermum sp. (Fig. 8.1). However, no localization was detected in Lemanea sp. Possibly the absence of localization in Lemanea sp., as in other algae tested (3.3, Table 3.13), was a result of either the inability to hydrolyze the substrate or a combination of the short incubation time required by the assay and low activity in the sample. Optimal cell bound PMEase activity in Batrachospermum sp. was at pH 10.3 (Fig. 8.1), which is similar to the significantly high pH optima found in hair-forming Rivulariaceae (Table 3.5 and 3.7).

Fig. 8.1 Localization of cell-bound PMEase activity in the hair cells of *Batrachospermum* sp. Scale bar = 10 µm.

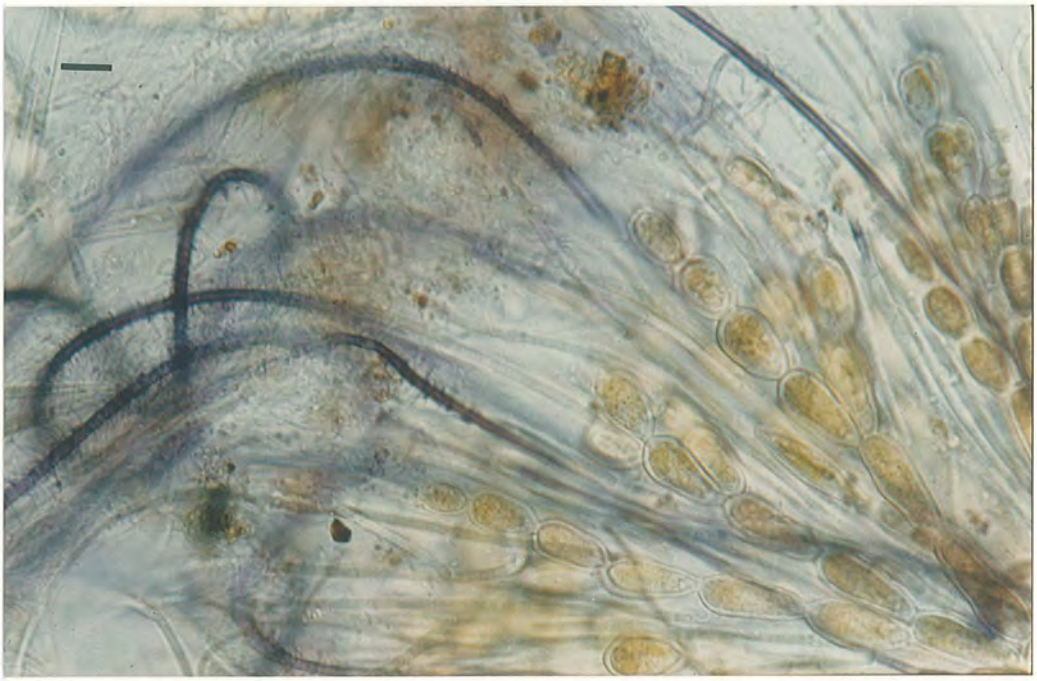
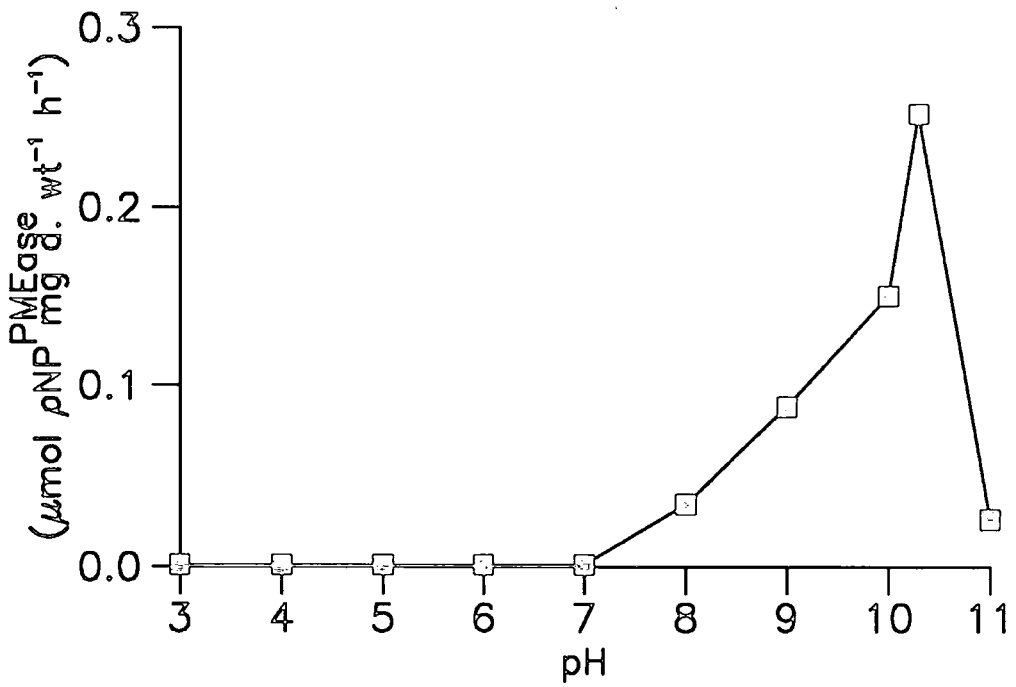


Fig. 8.2 Influence of pH on cell-bound PMEase activity in Batrachospermum sp.



CHAPTER 9

DISCUSSION

9.1 PHOSPHATASE ACTIVITIES AND ORGANIC PHOSPHORUS UTILIZATION IN 51 CYANOBACTERIA

All 51 cyanobacterial strains tested exhibited inducible cell-bound PMEase activity at either pH 10.3 and/or pH 7.6 (Table 3.10). Amongst these strains the Rivulariaceae were the most efficient at organic P utilization (Table 3.6) and generally had significantly higher cell-bound PMEase activities at pH 10.3 (Table 3.7).

Synechococcus compared with Rivulariaceae had significantly lower yields in all the P sources, except ATP (Table 3.6), and significantly lower cell-bound PMEase activities at pH 10.3 and pH 7.6 (Table 3.7). Five out of the seven Synechococcus strains were unable ($P = 0.001$) to utilize phytic acid (Table 3.6), and varying incubation conditions with phytic acid led to no increase in yield in any of the Synechococcus strains (3.3). Phytic acid is a major component of the colloidal phosphorus present in aquatic environments and is hydrolyzed by the group of phosphatases known as phytases (Mitchell and Read, 1981). As Synechococcus is a genus which is mainly planktonic it may be adapted to utilize low external concentrations of P_i and dissolved organic phosphorus (DOP) substrates typical of a planktonic environment. Pettersson (1980) suggested that Synechococcus strains in Lake Erken adapted to the low concentrations of organic P by increasing the substrate affinity (reducing K_m of their PMEases).

However, hair-forming Rivulariaceae were significantly more effective at utilizing phytic acid than non hair-forming Rivulariaceae strains (Table 3.6) and five out of seven Nostoc strains had high yields in phytic acid (Table 3.2). These strains may be adapted to environments where a major source of P is colloidal or P associated with high molecular weight compounds (Broberg and

Persson, 1988). Seven of the nine hair-forming strains were isolated from stream habitats (Table 3.10a) and Calothrix 550 was isolated from an environment where organic P in the March-May period (Livingstone and Whitton, 1984) reached 1.0 mg l^{-1} P. Possibly hair-forming strains may be adapted to stream environments with high concentrations of colloidal organic P.

Three hair-forming strains were unable to utilize ATP and the cultures became bleached (3.3), which suggests that ATP is toxic to these strains. Comparison of the utilization of ATP in hair-forming strains, showed that they were significantly less effective than non hair-forming strains at utilizing ATP (Table 3.2 and 3.6). Possibly ATP at high concentrations enters the hair cell resulting in disruption of cell metabolism and cell death. If this was the case organic P at high concentrations may also be hydrolyzed within the hair cell.

Rivulariaceae had significantly higher levels of cell-bound PMEase activity at pH 10.3 than the other taxa tested (Table 3.3 and 3.7), which is towards the upper end of the range observed for other cyanobacteria (pH 8-10: Healey, 1973; Ihlenfeldt and Gibson, 1975 and Healey and Hendzel, 1979). In hair-forming strains cell-bound PMEase activity significantly declined below pH 10.3 (Table 3.3 and 3.7). As cell-bound PMEase activity is significantly higher in Rivulariaceae strains (Table 3.7) it suggests that high concentrations of phosphomonoesters may also be a major P source along with phytates and colloidal P utilized by hairy strains.

Strains isolated from deepwater and paddy rice habitats were effective at hydrolyzing the phosphodiesters bis-ρNPP and DNA (Table 3.10a). Correlated with effective utilization of phosphodiesters are significantly higher levels cell-bound PDEase activity in deepwater rice strains compared to isolates from streams and ponds (Table 3.12). Phosphodiesters, such as nucleotides, have been identified as major pools of DOP in various environments (Broberg and Persson, 1988). Phosphodiesters may therefore be a major organic P fraction

available in deepwater and paddy rice habitats. If this is so, it may be one reason to explain why cyanobacteria are very successful in these habitats. Extracellular PDEase activity was only detected in six strains, all isolated from deepwater rice areas, whereas 48 strains had extracellular PMEase activity (Table 3.10). It is likely that PDEases are generally cellular and cell-bound enzymes, although there are apparently no comparisons of PMEase and PDEase activity for micro-organisms. PDEase activity compared with PMEase activity was always lower, which may suggest that there is less PDEase than PMEase enzyme present. However, as suggested by Kelly et al. (1975), the lower PDEase activities may be caused by the fact that bis-ρNPP is difficult to hydrolyze whereas ρNPP is easily hydrolyzed (1.66). Precaution must therefore be taken when comparing activities, as they can depend on the substrate used (1.66). Isolation of PDEases (1.661) in a wide variety of organisms proved that PDEase activity is attributed to completely different enzymes and is not the result of PMEases acting on phosphodiesters.

Extracellular PMEase activity at pH 10.3 was significantly higher in pond isolates compared to other physical environments tested (Table 3.12). In planktonic environments, such as ponds and lakes, the organic P levels may be more constant and less prone to fluctuations than in other environments. Possibly extracellular PMEases are more effective at utilizing organic P sources in planktonic environments compared to environments where phosphatases are likely to be carried away from the site of synthesis.

No major differences were found between strains originating from calcareous and non-calcareous environments in the utilization of organic P sources. However the mean rank of phosphatase activities were higher in calcareous isolates (Table 3.11b). Calothrix and Gloeotrichia were generally the most effective genera in the Rivulariaceae at utilizing organic P sources and were the genera with the highest phosphatase activities (Table 3.8 and 3.9).

Overall the 51 cyanobacterial strains have shown significant patterns of phosphatase activities and the utilization of various organic P sources in relation to various taxa and physical environments tested. However, further work on more taxa would be needed to conclude that the Rivulariaceae is the taxon most suited to habitats where organic P is present in high concentrations.

Localization of PMEase activity with naphthol AS-MX phosphate (3.3, Table 3.13), in the cyanobacterial strains tested, fell into two broad patterns. Distinct separation of PMEase activity on the hair and none in the remaining trichome was shown for seven hair-forming Rivulariaceae. In Calothrix parietina 184 and 550 the opposite staining pattern occurred. However, localization of PMEase activity in Calothrix 550 with BCIP showed localization of activity on hair cells, sheath and mucilage. It is possible that the PMEases located in hair cells of Calothrix 550 and 184 were unable to hydrolyze naphthol AS-MX phosphate. The second pattern was found in the remaining cyanobacterial strains where no distinct PMEase localization or specialization of cells was noted. PDEase activity, when detected in a 15 min incubation with β-naphthyl phenylphosphonate (2.733, 3.3), had no specific area of localization. The patterns of PMEase and PDease localizations are therefore in complete contrast. The differences in localization may suggest that PMEase and PDEase activities in the cyanobacterial strains studied are the result of different enzymes.

9.2 EFFECT OF ORGANIC PHOSPHORUS SOURCES ON PHOSPHATASE ACTIVITY AND HAIR FORMATION

Induction of phosphatase activity has been rarely investigated in algae (Aaronson and Patni, 1976). Calothrix 202, 550 and 603 (4.1) when grown in a range of organic P sources had the highest phosphatase activities in bis-pNPP and lowest activities in P_i. Differences in activities in the various

organic P sources were probably not caused by induction, but by a variation in cellular P values, i.e. bis-pNPP had lower cellular P values than P_i at the same time (day) in the growth curve (Fig. 4.1, 4.133, 4.134). Synthesis of cell-bound and extracellular PMEase activity started when cellular P values were between 0.6% - 1% dry wt (Fig. 4.1, Fig.4.3 and Fig. 4.4)).

Cell-bound PDEase activities, per unit biomass, in Calothrix 202 and 550 were highest at 4 d and reduced thereafter (Fig 4.7), which suggests that the synthesis of PDEases started when cellular P values were above 1% (Fig. 4.3). Possibly PDEases in these strains are constitutive enzymes synthesized independently of the P status. This is in direct contrast to PMEases, which are generally inducible enzymes where synthesis increases with P-deficiency (Healey, 1982).

Hairs were not observed in Calothrix 202 under various field and laboratory conditions, Calothrix 550 formed hairs under P-deficiency in both conditions and Calothrix 603 only formed hairs in the field (4.111). Calothrix 202 and 603 did not form hairs in any organic P source tested, although similar patterns of phosphatase activity compared to Calothrix 550 were reported (4.134). It was possible that hair formation in Calothrix 603 was inhibited by the composition of the medium (6.3, 9.4). Calothrix 202 had no detectable extracellular phosphatase activity. Possibly Calothrix 202 was adapted to a freshwater pool in Aldabra (Table 2.1) prone to fluctuations of organic P (Whitton pers. comm.) and the possession of extracellular PMEases may not have confered any advantage (9.1).

9.3 PROPERTIES AND LOCALIZATION OF CELL-BOUND AND EXTRACELLULAR PHOSPHOMONOESTERASES IN HAIR-FORMING RIVULARIACEAE

In Chapter 5 and 6 effects of environmental variables, ionic composition and localization of PMEase activity were tested in hair-forming freshwater Calothrix 253 and 550 (6.12). No extracellular PMEase activity was detected

in Calothrix 253 and no extracellular PDEase activity was detected in either strain (3.3).

Calothrix 253 and 550 had typical inducible PMEase activity increasing with the onset of P-deficiency and synthesis of enzyme was inhibited with addition of P_i (McComb et al., 1979). Both algae had high pH optima for cell-bound PMEase activity at pH 10.0-10.2 (5.23, Fig. 5.2) and pH 12.2 (6.13, Fig. 6.2), respectively. Temperature optima were also high for Calothrix 253 and 550 at 50°C (6.13, Fig. 6.2) and 45°C (5.13, Fig. 5.1), respectively. These results suggest that maximum activities reported in the laboratory (>10 μmol ρNP mg dry wt^{-1} h^{-1}) would not be achieved in field conditions, e.g. Calothrix 550 originates from an environment with a $\bar{x}$ pH of 7.8 (Grainger et al., 1989), a pH value at which laboratory activity is about 40% of the maximum.

Temperature optima in Calothrix 550 (45°C) were above temperatures likely to be encountered in its original environment (Grainger et al., 1989), and for most of the year activity may be expected to be less than 30% of the maximum. It is difficult to comment on Calothrix 253 as there are few data on its original environment (isolated by J. Komárek, from the upper part of a mangrove root in Cuba, see 6.1). However it is unlikely that a temperature of 50°C or a pH of 12.2 would be reached.

Calcium had the greatest stimulatory effect in Calothrix 253 (5.33, Fig. 5.3) and 550 (6.13, Fig. 6.2b), which matches the results in other cyanobacteria (Glew and Heath, 1971; Healey, 1973 and Doonan and Jensen, 1980). Concentrations of Ca are likely to reach levels in the environment (>1 mM, 5.33), which are sufficient to increase phosphatase activities. In Calothrix 253, Ca was required for activity, suggesting this element plays a vital role in enzyme structure. Possibly Ca is an integral part of the active site of the enzyme in Calothrix 253 as in Micrococcus sodenensis (Glew and Heath, 1971). PMEase activity in both strains was inhibited by Mg, although it has been reported to be stimulatory in E. coli (Schlesinger et

al., 1969), Aspergillus niger (Dorn, 1968) and Pseudomonas aeruginosa (Day and Ingram, 1973). The inhibitory effect of EDTA on PMEase activity in Calothrix 550 (5.33) and Nostoc commune (Whitton et al., in press) was similar to that found in other studies (Whitt and Savage, 1988), which suggests that one or more metals are needed for PMEase activity in algae as they are in heterotrophic bacteria and mammalian PMEases (Whitt and Savage, 1988).

From the above information it is likely that cyanobacterial PMEases are similar to PMEases in heterotrophic bacteria in that they are metallo-enzymes; have high temperature optima, are inhibited by P_i, are generally alkaline and inducible and have an overall similar structure. However, the major differences from heterotrophic PMEases are the high pH optima (>pH 10.0) and the common stimulatory effect of calcium (Doonan and Jensen, 1979). It is possible that the PMEases are glycoproteins bound to a surface (5.33) unlike bacterial PMEases which are commonly periplasmic enzymes (1.62).

Properties of PMEase in N. commune (Whitton et al., in press) contrast with most of the properties of PMEase activity in hair-forming Rivulariaceae strains. N. commune had a lower pH optimum (pH 7.0) and a lower temperature optimum (32°C), which is very similar to the properties of typical heterotrophic bacterial PMEases (1.62). Five Nostoc strains tested (3.1) also had lower activities at pH 10.3 versus pH 7.6 (Table 3.9). N. commune was able to utilize all of the organic P sources except phytic acid (Whitton et al., in press); a similar result was also found in Nostoc 201 (Table 3.2 and 3.8). The above result in these two Nostoc strains, which originated from similar environments as hair-forming Rivulariaceae strains (Table 2.1), is the direct opposite as hair-forming Rivulariaceae which are effective at hydrolyzing phytic acid (9.1). The ability to hydrolyze phytic acid may be very important to Rivulariaceae as their environments may have a large proportion of available phytic acid. Herbes et al. (1975) suggested that phytic acid may be an important organic P source in freshwater environments.

Effects of ions on phosphatase activity of N. commune were similar to those of Calothrix 253 and 550.

Evidence that hairs are an important site of phosphatase activity in Calothrix 550 was obtained by using a cellular fraction consisting of detached hairs (5.32). This fraction showed high activity with ρNPP, BCIP and the phosphodiester bis-ρNPP, but no activity with naphthol AS-MX phosphate (cf. 9.1). This experiment concluded that hairs in Calothrix 550 contained high levels of cell-bound PMEase and PDEase activity. The procedure for detachment and collection of hairs would remove any contaminating cellular phosphatases. Localization of activity on the hairs in Calothrix 550 was achieved with BCIP (2.731, 5.33, Fig. 5.4).

The differing responses of Calothrix 550 to BCIP in shaken vials and under a coverslip may be due to differing availabilities of O_2. Staining by BCIP results from two separate reactions (Coston and Holt, 1958). BCIP is first hydrolyzed, releasing one molecule of soluble, colourless indole and one molecule of P_i; this is followed by an oxidation step, which leads to the formation of the insoluble blue indigoid pigment, 5-bromo-4-chloro-3-indigo, localized at the site of PMEase activity (Fig. 5.4). Staining is therefore dependent on enzyme activity and presence of O_2 (2.731). The initial O_2 concentration is about 15 mM (at 32°C), whereas BCIP is supplied at 1 mM. Released indole will scavenge O_2. Unless photosynthetic O_2 evolution compensates for this, it will lead to a reduced concentration under the coverslip, whereas the saturation concentration will be maintained in the shaken vial. It is likely that under the coverslip indole enters the hair cell initially and is then oxidized in the cytoplasm by O_2 resulting from photosynthesis.

Further proof of localization of PMEase activity in hair cells was envisaged using antibodies obtained from purified PMEase. PMEases were partially purified from the extracellular fraction on a non-denaturing

polyacrylamide gel (5.33). Four separate PMEase bands were detected with BCIP (Fig. 5.4a); however these bands were not retrievable by physical means (5.32) and lost activity when left on polyacrylamide gels for longer than 12 h. PMEase bands 1, 2 and 4 (Fig. 5.44a,b), which were the major PMEase bands present, were bound to carbohydrate (Fig. 5.4b). As PMEases were bound to carbohydrate, except for band 3, it is likely this prevented removal of the PMEases by mechanical means.

It is difficult to determine whether the different bands are structurally different extracellular PMEases as the association of proteins, a common occurrence on non-denaturing gels, may result in bands of different molecular weights. Different banding patterns may also be caused by carbohydrate, of various molecular weights, bound to PMEase. If antibodies were raised against PMEase in Calothrix 550 it is possible the epitope would be on the carbohydrate part of the PMEase, which would negate the use of antibodies as a useful tool for localizing PMEase activity in hair cells. If PMEases in Calothrix 550 are bound to carbohydrate it raises two points of discussion:
i) Are PMEases in Calothrix 550 physically bound to carbohydrate and if so are they "truly" extracellular enzymes?
ii) Or, alternatively, are extracellular and cell-bound PMEases in Calothrix 550 glycoproteins?

The most convincing evidence of hair cells as sites of PMEase activity was gained using the azo-dye naphthol AS-MX phosphate (2.732) in Calothrix 253 (6.13, Fig. 6.1) and other hair-forming Rivulariaceae (3.3, Table 3.13). Azo-dye staining indicated that there were no detectable PMEase activities associated with vegetative cells and there was a sharp transition from vegetative cells with no activity to hair cells with PMEase activity. As suggested previously, hairy Rivulariaceae are adapted to environments where the P is available in pulses of high external concentrations of organic phosphoesters and organic colloidal P (phytases, Herbes et al., 1975). If

the phosphatases in these cyanobacteria are located solely on the hairs it suggests that the hairs are adapted to utilize these high concentrations of organic P. Therefore, if hairy Rivulariaceae are present it is possible to use these organisms as environmental indicators of the P dynamics/cycling of particular environments.

9.4 INFLUENCE OF SALINITY ON HAIR FORMATION IN THE RIVULARIACEAE

Hair formation in Calothrix 253 is suppressed when grown in 90 mM NaCl (20% seawater salinity, Chapter 6). Hair formation in Calothrix 253 can occur only when the trichomes are P limited and grown under low salinity. However, growth does occur in media up to 30% of the salinity value of seawater. Hair formation can thus be brought about in two ways: by reduction of P_i content during growth in freshwater medium or by reduction of P_i content during growth in saline medium, followed by transfer to freshwater medium (Fig. 6.4). Under the latter conditions hair formation was largely synchronised. This occurrence was used as an effective tool to compare organic P hydrolysis and phosphatase activities in Calothrix 253 with and without hairs (6.1).

In spite of the much lower cell-bound PMEase activity in saline medium (Table 6.2), the yield with β-glycerophosphate was similar in saline and freshwater medium, but with all other organic P sources, the yield was much lower. Azo-dye staining (naphthol AS-MX) indicated that there was no detectable cell-bound PMEase activity associated with any part of the cell wall, sheath or mucilage in material grown in saline medium. As there was no evidence of cell rupture it proposes that hydrolysis of organic P may take place inside the cell (cellular PMEases), as suggested for the hydrolysis of ATP by three hair-forming Calothrix strains (9.1). This would provide an alternative explanation for the presence of high levels of cellular PMEase in P-deficient Anabaena PCC 7119 reported by Marco and Orus (1988), who suggested that the alga tried to relieve P-deficiency by hydrolyzing its cellular

phosphates. If this situation applies to cultures grown in freshwater medium, the intense PMEase activity associated with the hair surface may arise because hair cells are especially well suited to hydrolyze pulses of high concentrations of organic P. Evidence to support the role of hairs in making use of pulses of high concentrations of NH_4-N has been provided for Ceramium rubrum (D'Elia and DeBoer, 1978; DeBoer and Whoriskey, 1983) and it has been suggested that this may occur with respect to organic P in other hair-forming eukaryotic algae (Whitton, 1988).

Added Ca during growth with 67.5 mM NaCl (15% seawater salinity) initially enhanced hair development (6.13) and then led to culture lysis. Neither mannitol nor sorbitol (6.13) had any effect on hair formation, indicating that suppression of hair formation by NaCl is not an osmotic effect. These observations suggest that hairs in Calothrix 253 may be unable to control uptake of one or more potentially toxic ions such as Na. Apte and Thomas (1986) suggested that Na exclusion formed the basis of cyanobacterial salt tolerance, so the suppression of hair development at higher salinities (>15% seawater salinity) may be an adaptation to avoid Na toxicity. Ca possibly effects PO_4 uptake/metabolism resulting in P-deficiency and enhanced hair development at 67.5 mM NaCl. Therefore, increased hair development would result in uncontrolled uptake of Na and culture lysis.

Many other members of the Rivulariaceae occurring in intertidal environments can form obvious hairs and there is no indication from the taxonomic literature that the tendency to form hairs is less pronounced in such environments. Therefore, an explanation is still needed as to why hair formation in Calothrix 253 does not take place above 15% seawater salinity. A possible ecological explanation may be put forward based on its microhabitat on a mangrove root. There may be no physiological advantage in possessing hairs when submerged in saline water or under normal emergent conditions. However, prolonged rain might not only change the root surface to a more

freshwater environment, but lead to a pulse of organic P. If hairs are especially adapted to deal with pulses of organic P, the rapid development of hairs in Calothrix 253 when transferred from saline to freshwater conditions may be a further adaptation to its particular habitat. A somewhat similar explanation can be put forward for Calothrix 690 (6.2), which was isolated from the edge of a Saudi Arabian waterfall, and showed a similar influence of salinity on hair formation.

A further similarity between Calothrix 253 and 690 was the unusually high PMEase pH optima of pH 12.2 (Fig. 6.2) and pH 11.8, respectively (Fig. 6.5). Is it likely that the effect of Na and the high pH optima are somehow linked?

A reduction in Na from 1.67 mM-0.022 mM in Chu 10D-N (6.3) resulted in a marked increase in trichome length and vacuolation in Calothrix 764 and Gloeotrichia 281 and 613. Previously these strains formed hairs in field conditions, but not under laboratory conditions. The changes in trichome morphology resembled hair formation. Trichomes that responded to reduced Na in Calothrix 764 also showed a differential staining pattern with activity localized on the apical part of the trichome, similar to the pattern of staining in true hair-forming strains (Fig. 6.6, 9.1). As only some trichomes showed this pattern, it is likely that trichomes which were P-deficient and formed in the presence of low Na developed this particular response. It is suggested that previously hairs did not form in these strains, because the relatively high levels of Na present in standard Chu 10D inhibited hair formation. Therefore, if further research was to take place on hair-forming algae it would be advantageous to reduce the Na level to 0.022 mM or at least a Na concentration below 1.67 mM (6.3). The inhibition of hair formation in these strains is similar to the results in Calothrix 253 and 690, but the concentrations of Na needed for inhibition of hair formation were much lower. As the effects of Na occur at a much lower concentration in Calothrix 764 and Gloeotrichia 613 it may be because these strains are adapted

to a deepwater rice habitat (Sonargon, Bangladesh) where elemental concentrations are low and the Na level in the environment is below 1 mM (Whitton et al., 1988). No comment can be made for Gloeotrichia 281, as no data are available on the nutrient concentrations in its original environment. As two of these strains are adapted to an environment with low external concentrations of Na (< 1mM), it is possible that they do not possess or require an efficient Na extrusion mechanism (Apte and Thomas, 1986).

9.5 PHOSPHATE UPTAKE IN HAIR-FORMING RIVULARIACEAE

High levels of cell-bound PMEase activity, hair formation, originating from streams associated with pulses of high concentrations of organic P levels are factors associated with the success in growth of hair-forming Rivulariaceae. These factors will result in the rapid hydrolysis of high concentrations of phosphoesters by hair cells and the subsequent release of P_i at high concentrations. Therefore, if these algae are adapted to these particular environments, it is likely that they possess a rapid P_i uptake system adapted for efficient uptake of P_i at local high external concentrations. If this is not the case large amounts of P_i may be lost downstream to other competing organisms.

As hair cells are the sites of PMEase activity and subsequently the site where high concentrations of P_i are released it is likely that the hair cells are also the sites of P_i uptake at high external concentrations.

Comparison of versions of freshwater (+ hair) and saline (- hair) Calothrix 253 was used as a method for evaluating P_i uptake in a P-deficient species with and without hairs (7.1). Results from Chapter 7 showed that freshwater Calothrix 253 (Fig. 7.2a) had high P_i uptake values and associated high K_m (low affinity), high V_{max} (high velocity) and ratio of K_m to V_{max} values, which are values adapted for P_i uptake at high concentrations (Table 7.2). However, saline Calothrix 253 (Fig. 7.2a) had negligible P_i uptake and had a

low K_m (high affinity), V_{max} (low velocity) and ratio of K_m to V_{max} values and therefore has a P_i uptake system adapted to low P_i concentrations (Table 7.2). As both versions were P-deficient (Table 7.2) the major difference in P_i uptake can only be explained by the presence or absence of hairs. A similar high velocity nutrient uptake system was described for Ceramium rubrum (DeBoer and Whoriskey, 1983), where the hair cells in this alga possessed an uptake system with a low affinity (high K_m) and high velocity (high V_{max}) adapted to take up high external concentrations of NH_4-N.

The above conclusions indicate that hair-forming Rivulariaceae have both the high level of PMEase activity and the high velocity P_i uptake system required to hydrolyze high concentrations of organic P and then take up the resultant high concentrations of P_i released.

In Calothrix 550 the V_{max} of P_i uptake was the second highest reported for cyanobacteria next to 1.8 μmol P mg^{-1} h^{-1} in Anabaena flos-aquae (Healey, 1982). The high V_{max} and K_m values (Table 7.2) in hairy Calothrix 550 is further evidence to support the theory that hairy Rivulariaceae are adapted to P_i uptake at high concentrations. In comparison Synechococcus may be adapted to utilize low concentrations of DOP (Pettersson, 1980) and P_i in the environment (3.3, 9.1) and may have a P_i uptake system with a high affinity (low K_m) and a low velocity (low V_{max}).

20 μM DCMU and dark reduced P_i uptake, but only between 0-15 μM PO_4, in hairy Calothrix 253 and 550. Possibly two P_i uptake systems are present, one active uptake system associated in the vegetative cells, which is adapted to transport P_i at low external concentrations (0-15 μM PO_4). The second P_i uptake system is one with a high K_m and V_{max}, associated with the hair cells, and is adapted to transport P_i at high concentrations. This uptake system is similar to the two P_i uptake systems (1.7) described in E. coli (Rosenberg et al., 1978; Willsky and Malamy, 1980), with the inducible P_i uptake system,

adapted to transport P_i at high external concentrations, associated with hair cells in cyanobacteria.

Pi uptake in Calothrix 253 increased 100% at pH 9.0, which is above the pH optima, of pH 7-7.6, quoted for P_i uptake in algae (Healey, 1982). This result compares directly with the high pH optima for PMEase activity in Calothrix 253. Possibly the high pH optima of PMEase activity and P_i uptake at high concentrations in the hair cells are linked in Calothrix 253. It is possible that the site of the PMEase is directly linked to the site of the pore protein associated with P_i uptake (Torriani-Gorini et al., 1987). Also Ca^{++} stimulated P_i uptake in Calothrix 253 as it did with PMEase activity (Fig. 6.3a), which further backs up the hypothesis that P_i uptake and PMEase activity occur at the same site on the hair cell surface. Mg^{++} above 0.1 mM was a potent inhibitor of P_i uptake in Calothrix 550; possibly this is a further adaptation to its original habitat where magnesium is present at very low concentrations (Livingstone and Whitton, 1984).

9.6 LOCALIZATION OF PHOSPHOMONOESTERASE ACTIVITY IN HAIR-FORMING EUKARYOTIC ALGAE

The eukaryotic hair-forming algae Draparnaldia sp. and Batrachospermum sp., collected from environments with high concentrations of organic P, had distinct localization of cell-bound PMEase activity on the hair (Fig. 8.1). This is the first report using naphthol AS-MX phosphate to localize PMEase activity on eukaryotic hair cells, possibly indicating that this technique may be useful to localize PMEase activity in a wide range of organisms. However organisms with low PMEase activity, such as Lemanea sp. (8.3), may be unable to hydrolyze sufficient amounts of naphthol AS-MX phosphate in the 15 min incubation period (2.732). If there is low PMEase activity, BCIP (2.731) may be used to localize activity, as there is no limit on incubation time with this substrate. BCIP can effectively detect PMEase activity in transparent

cells (Fig. 5.5) or gels (5.4) and has been used to localize PMEase activity in higher plant roots (A. Slatter, pers. comm.). However, it is worth noting that the blue colour of the indigoid is difficult to distinguish from the photosynthetic pigments in cyanobacteria (5.33).

From the results of staining (8.3) and previous research on hair-forming eukaryotic algae (Gibson, 1986, Gibson and Whitton, 1987 and Whitton, 1986), it is likely that the hypothesis of the adaptation of hair-forming Rivulariaceae to environments where P is present in pulses of high concentrations of organic P may also encompass hair-forming eukaryotic algae (Whitton, 1988). P_i uptake experiments were not performed on these algae, although research on the hair-forming eukaryotic alga Ceramium rubrum (DeBoer and Whoriskey, 1983) suggested that low affinity high velocity nutrient uptake occurred only in hair cells.

SUMMARY

1) All 51 cyanobacterial strains studied exhibited inducible alkaline PMEase activity at either pH 10.3 and/or pH 7.6. In all cases the level of cell-bound PDEase activity measured was lower than cell-bound PMEase activity. Extracellular PDEase activity was only detected in six strains, whereas extracellular PMEase activity was detected at either pH 10.3 and/or pH 7.6 in 48 strains.

2) Rivulariaceae was the most effective large taxon studied at organic P utilization ($P = <0.01$) and Synechococcus the least effective large taxon ($P = <0.01$). Synechococcus strains were unable to utilize phytic acid ($P = <0.001$). The addition of ATP resulted in the bleaching of three hair-forming strains and significantly reduced yields compared to non hair-forming Rivulariaceae ($P = <0.05$).

3) Deepwater rice strains had significantly higher levels of PDEase activity than pond ($P = <0.01$) and stream isolates ($P = <0.01$). It is suggested that these strains may originate from environments where phosphodiesters are a major source of organic P.

4) Strains originating from still water habitats and surface waters had significantly higher levels of extracellular PMEase activity than deepwater rice ($P = <0.01$), paddy rice ($P= <0.05$) and stream isolates ($P = 0.05$).

5) Rivulariaceae had significantly higher levels of cell-bound PMEase activity at pH 10.3 than non-Rivulariaceae and Synechococcus strains tested. Hair-forming Rivulariaceae were significantly more effective than non hair-forming Rivulariaceae at hydrolyzing phytic acid ($P = <0.05$). Cell-bound

PMEase localization using either BCIP and/or the azo dye naphthol AS-MX phosphate showed that PMEase activity was confined to the hair cells in all nine hair-forming Rivulariaceae strains studied. Cell-bound PMEase activity was also localized solely on the hair cells of Batrachospermum sp. and Draparnaldia sp. using naphthol AS-MX. Eight of the nine hair-forming Rivulariaceae strains studied originated from stream environments prone to pulses of organic P.

6) P_i uptake experiments using freshwater Calothrix 253 and 550 showed that both strains had high K_m values of 12.930 and 14.83 µM PO_4 (low affinity) and high V_{max} values at 0.852 and 1.757 µmol P mg dry wt^{-1} h^{-1}(high velocity), respectively. Uptake at high P_i concentrations (>15 mM PO_4) was confined to hair cells in Calothrix 253 and was not affected by the absence of light or by 20 µM DCMU.

7) Addition of various organic P substrates did not modify inducible phosphatase activities or hair formation in Calothrix 202, 550 and 603. Cell-bound PMEase activity in Calothrix 202, 550 and 603 commenced when cellular P values fell below the range 0.6 - 1.0 % cellular P (P as % dry wt).

8) Optimal cell-bound PMEase activities in hair-forming Calothrix 253 and 550 were at 50°C and 45°C and pH 12.2 and 10.2, respectively, which were conditions unlikely to occur under normal field conditions. The influence of 11 ions on PMEase activity in Calothrix 253 and 550 was tested. Of the 11 ions Ca was found to be the only ion most likely to reach concentrations in the environment which may influence phosphatase activities in both strains.

9) No significant increase in extracellular PMEase activity occurred when Calothrix 550 material was exposed to trichloroethane (0.1 mM - 100 mM), 20% sucrose or lysozyme treatment. These all suggest that the PMEase enzyme is bound to a surface.

10) The response of cell-bound and extracellular PMEase activities in Calothrix 550 to environmental variables was the same and it is suggested that the two fractions contain the same enzyme. Partial purification of the extracellular PMEase fraction in Calothrix 550 resulted in the detection of four PMEase bands using BCIP. Three of the four PMEase bands were bound to carbohydrate and it is possible that certain PMEases in Calothrix 550 may be glycoproteins.

11) Na, above 67.5 mM (15% seawater salinity), had a marked inhibition on hair formation in Calothrix 253 and 690 and had a similar effect at 1.67 mM Na in Calothrix 764 and Gloeotrichia 281 and 613. Transfer of P-deficient saline Calothrix 253 to freshwater medium led to the rapid (7 h) synchronised formation of hairs and the subsequent detection of cell-bound PMEase activity in the newly formed hairs.

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Phosphomonoesterase activity of the cyanobacterium (blue-green alga) *Calothrix parietina*

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Abstract

Cellular and extracellular phosphomonoesterase activities were compared in *Calothrix parietina* D550, a strain whose original environment has been studied in detail. Activity in both fractions became detectable at about the same stage in batch culture. Differences in the influence of environmental factors between the two were slight, suggesting a common origin. The optimum temperatures for cellular and extracellular activities were 40°C and 30°C, respectively, and the upper limits for detectable activity were 80°C and 65°C. The pH optimum for both cellular and extracellular activity was 10.0–10.2. When P-limited cultures were tested with *p*-nitrophenyl phosphate (*p*NPP) as substrate, K_m values for cellular and extracellular activities were 43 and 33 μM *p*NPP, respectively. Eleven ions were tested for their influence on activity. In most cases the effect was low or negligible at concentrations likely to be present in nature or freshwater laboratory media. Where obvious effects occurred, these were usually apparent at lower concentrations with extracellular than cellular activity. One mM Ca led to a 40% increase in extracellular activity in comparison with 0.1 mM Ca, but had no effect on cellular activity. However, inorganic phosphate, which had a marked inhibitory effect at concentrations above 10 μM, brought about a similar response with cellular and extracellular activities (approximately 60% decrease with 100 μM).

Introduction

Micro-organisms with phosphatase activity are able to hydrolyse phosphate from a variety of organic phosphorus compounds (Healey, 1982; Torriani-Gorini *et al.*, 1987). Phosphomonoesterase activity appears to be very widespread among P-limited cyanobacteria, though not universal (Healey, 1982). Among eighteen strains tested by Doonan and Jensen (1980), all showed activity, this being clearly inducible in twelve strains. The most detailed studies have been made on *Anabaena variabilis* (Healey, 1973; Healey and Hendzel, 1975) and *Plectonema boryanum* (Doonan and Jensen, 1977, 1979, 1980).

Cell-bound enzyme of *P. boryanum* differed from cell-free enzyme obtained by breaking open the cells in its response to several ions (Doonan and Jensen, 1979). For instance, cell-bound phosphatase was inhibited markedly by zinc at all concentrations, whereas the effect was not detected with cell-free enzyme in assay medium with zinc below 2 mM. Some phosphomonoesterases are tightly retained in the cell wall and lose activity when released (Doonan and Jensen, 1977), whilst others are active when released into culture medium (Healey and Hendzel, 1975). Ten of the strains studied by Doonan and Jensen (1980) showed extracellular activity.

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There is evidence that Rivulariaceae occur in environments where organic phosphorus is an important source of phosphate, and that field populations exhibit marked phosphatase activity (Whitton, 1987). The enzyme is apparently always inducible in this family and activity of P-deficient *Gloeotrichia echinulata* is among the highest recorded (Fitzgerald and Nelson, 1966; Healey, 1982) for cyanobacteria. A strain of *C. parietina* (D550) isolated from an upland stream, where it is sometimes a co-dominant (Holmes and Whitton, 1981), shows marked cell-bound and extracellular phosphatase activity in P-deficient culture. In batch culture phosphatase activity commences at about the same time as hairs develop (Livingstone *et al.*, 1983); evidence from staining suggests that much of the phosphatase activity is associated with the surface of the hair.

The aim of the present study was to establish to what extent phosphomonoesterase activities in this strain of *C. parietina* resemble those reported for other cyanobacteria and to what extent cellular and extracellular activities differ in their response to environmental factors.

Materials and methods

The organism used was an axenic clonal strain of *Calothrix parietina* Thur. (Durham culture 550). The stream from which the strain was isolated combines drainage from peat and limestone, with slight enrichment by zinc from earlier mining activity; most of the filtrable phosphate is in the organic fraction (Livingstone and Whitton, 1984).

The organism was grown in batch culture at 25°C and 60 μmol photon m^{-2} s^{-1} PAR, with the inoculum at *c* 10 mg l^{-1}. The medium was modified from the number 10 formula of Chu (1942): *viz* Na, *c* 1670 μM; K, 57.3 μM; Mg, 100 μM; Ca, 243 μM; P, 31 μM; S, 100 μM; Cl, 532 μM; B, 11.5 μM; Co, 0.0373 μM; Ni, 0.0338; Mn, 2.28 μM; Fe, 8.97 μM; Cu, 0.0789 μM; Zn, 0.193 μM; and Mo, 0.00276 μM. EDTA (0.09 mM) was used as chelating agent and 2.5 mM HEPES as buffer, with pH adjusted to 7.0 with NaOH. The Na concentration given above is that subsequent to adjusting the pH.

Cultures were harvested at a late growth stage (28 d) when markedly P-limited. Cellular material was obtained by centrifugation at 8,000 x *g* for 20 min, washed twice and resuspended in assay medium (see below). Cultures were homogenised using a series of sterile syringes with needles and then sonicated at 4°C using an MSE Soniprep 150 at an amplitude of 26 μm for 3 min. Most assays of extracellular activity were based on material harvested on one occasion and subsequently stored. Medium was separated from cellular material using eight layers of muslin. Three litres were rotary evaporated to 150 ml, centrifuged at 8,000 x *g* for 20 min to remove further debris and the supernatant dialysed against the assay medium, with three changes over 24 h. Storage over periods up to 7 d at 4°C and for longer periods (up to 8 months) at −20°C led to no detectable change in activity.

A fraction consisting almost entirely of detached hairs was obtained by sonicating a washed culture, centrifuging at a slow speed (500 x *g*) for 10 min and retaining the supernatant which was then centrifuged at 8,000 x *g* for 20 min and the pellet retained. This showed that at least 95% of the contents consisted of hairs.

Standard assays were carried out at pH 10.3 in medium buffered with glycine (50 mM) and NaOH and further differing from culture medium in the following: phosphate replaced by Cl (raising Cl to 564 μM); HEPES omitted; and Na raised to 36 mM. The homogenate (20 μl; cellular fraction), culture supernatant (extracellular fraction) or inorganic medium (controls) were pipetted into each of 96 wells containing 70 μl buffer. During the pipetting, the homogenate was agitated with a magnetic stirrer to reduce flocculation. Then 140 μl *p*-nitrophenyl phosphate (*p*NPP) suspended in assay medium was added to give a final concentration of 250 μM. The reaction proceeded at 32°C for 15 min or until yellow coloration was detected. Incubation was carried out without agitation, as agitation had no detectable effect during assay periods up to 30 min. Activity in the standard assay was terminated by the addition of NaOH to give a final concentration of 1.5 M. Measurements were made at 405 nm using a multiscan plate reader (Titertek). Activity is reported as μmol *p*NPP hydrolysed mg dry wt^{-1} h^{-1}.

To test the effect of temperature on phosphomonoesterase activity, aliquots of homogenate or medium were pre-incubated for 30 min at 5 degree intervals between 5°C and 85°C. Deviations in pH from 10.3 due to temperature were compensated for by the addition of NaOH or HCl. Temperatures above 85°C could not be tested directly because of the spontaneous hydrolysis of *p*NPP; the ability of the enzyme to tolerate temperatures above 85°C was tested by again lowering the temperature at 32°C and then testing with *p*NPP. The influence of pH on activity was tested using a range of buffers (Table 1). Two different buffers were tested at each pH value. Similar results were obtained with each buffer, except for pH 9.0 (glycine much higher than 2-amino-2-methyl-1-propanol) and pH 11.0 (3-cyclohexylamino-1-propanesulphonic acid much higher than Na_2CO_3 — $NaHCO_3$).

The effects of various substances on phosphomonoesterase activity were determined using 0, 0.01, 0.1, 1 or 10 mM concentrations in the assay mixture. The ions/molecules tested were Na^+, K^+, Mg^{++}, Ca^{++}, Fe III-chelate, Co^{++}, Cu II, Zn^{++}, phosphate, borate and molybdate. Mn was not tested, because of precipitation at pH 10.3. Cations were added as the relevant chloride or sulphate; NaOH used for buffering glycine was replaced by KOH in the case of the Na assay. Anions were added as the sodium salt. The dependence of phosphomonoesterase activity on the concentration of *p*NPP was described on the basis of Michaelis-Menton kinetics. To determine the K_m a Lineweaver-Burk plot was constructed.

The influence of EDTA on phosphomonoesterase activity was tested in two ways: *viz* its inclusion during an assay (medium, substrate and EDTA at 0.09, 1, 10 or 20 mM), and washing followed by resuspension in assay medium. For the latter, a 28-d-culture was centrifuged and washed twice, resuspended in

medium, or medium with increased EDTA (1, 10 or 20 mM) for 30 min, washed twice again to remove EDTA and then assayed for phosphomonoesterase activity (in medium). The presence of 20 mM EDTA reduced the pH of the assay medium to 9.78.

Carbohydrate in the medium from 28-d-cultures was assayed by the method of Dubois *et al.* (1956) in order to establish whether there is a correlation between phosphomonoesterase activity and the carbohydrate. Localization of enzyme activity was studied using various methods likely to release phosphomonoesterase, such as trichloroethane, 20% sucrose, and lysozyme-treated material previously exposed to 20% sucrose (Ingram *et al.*, 1973). Localization was also tested by microscopy using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as an organic P substrate (Coston and Holt, 1958; Holt and Withers, 1958). Filaments were washed three times, resuspended in 1 mM BCIP in the assay medium at pH 10.3, left for 15 min at 32°C and again washed three times.

Results

Cellular and extracellular phosphomonoesterase activities were first detected at a similar stage (day 7) during growth in batch culture. Activity in the medium from 28-d-cultures ranged from 10—20% of that in cellular material taken from a similar volume of culture (Figure 1).

The effects of temperature on cellular and extracellular activity were different (Figure 1), with optima at 40°C and 30°C, respectively, and maximum temperatures with detectable activity at 80°C and 65°C, respectively. When activity was assayed at 32°C following incubation for 30 min at elevated temperatures, activity was detectable in both fractions which had been incubated at 85°C (0.168 and 0.067 μM *p*NPP mg dry wt^{-1} h^{-1}), but was not detectable at 90°C. Incubation of extracellular enzyme with 10 mM Ca at temperatures in the range 65°C to 75°C led to no greater increase in activity than expected from the enhancing effect of Ca (see below). There was no difference in activity between dark and light (60 μmol photon m^{-2} s^{-1}) for either cellular or extracellular fractions. The pH optima for cellular and extracellular activities were between pH 10.0 and 10.2 (Figure 2).

The responses of cellular and extracellular activity to the eleven ions/molecules are shown in Figure 3; in a number of cases extracellular activity showed a slightly greater response. Calcium had the greatest stimulatory effect, with a 50% increase in activity at 10 mM for both systems. Slight enhancements of extracellular activity occurred with 10 μM K and 0.01—0.1 mM Zn, but cellular activity showed little, if any, response. Phosphate at 0.01 mM had no inhibitory effect, but in the range 0.1—10 mM had the greatest effect of any ion. For both cellular and extracellular activity, molybdate had a slight inhibitory effect at 1 mM and a marked inhibitory effect at 10 mM; Mg, Zn and borate also had marked effects at 10 mM. Comparisons were also included of the widely used BG11 medium (omitting combined nitrogen and phosphate) *versus* standard assay medium.

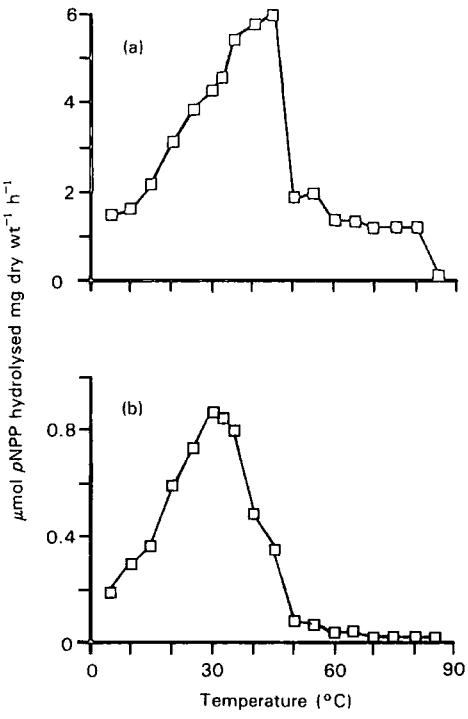

Figure 1 Influence of temperature on cellular and extracellular phosphomonoesterase activity in *C. parietina* D550 shown by hydrolysis of *p*NPP. Extracellular activity is related to the dry weight of alga from which the material was harvested. Note that the scale for cellular activity (▣) is ten times that for extracellular activity (□).

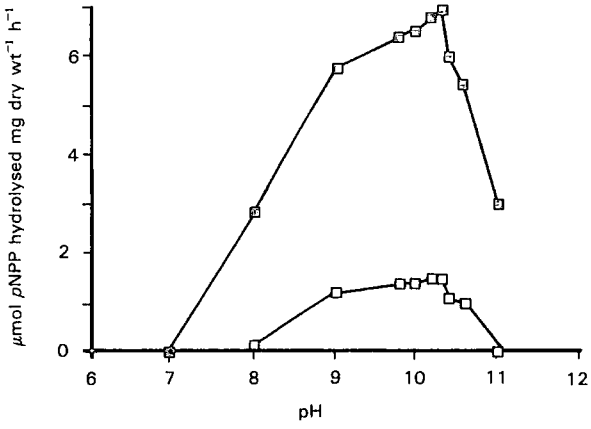

Figure 2 Influence of pH on cellular (▣), and extracellular (□) phosphomonoesterase activity in *C. parietina* D550 shown by hydrolysis of *p*NPP. Results shown are based on the higher of the activities found with the two buffers tested (Table 1) for each pH value.

Table 1 Buffers used for testing the effect of pH on cellular and extracellular phosphomonoesterase activity in *C. parietina* D550

pH	Buffer A	Buffer B	Buffer giving higher activity: Cellular	Extracellular
7.0	DMG – NaOH	HEPES – NaOH		
8.0	TES – NaOH	HEPES – NaOH	TES	TES
9.0	AMeP – NaOH	Glycine – NaOH	Glycine	Glycine
9.8	AMeP – NaOH	Glycine – NaOH	Glycine	Glycine
10.0	AMeP – NaOH	Glycine – NaOH	Glycine	Glycine
10.2	AMeP – NaOH	Glycine – NaOH	Glycine	Glycine
10.3	AMeP – NaOH	Glycine – NaOH	Glycine	Glycine
10.4	AMeP – NaOH	Glycine – NaOH	Glycine	AMeP
10.6	AMeP – NaOH	Glycine – NaOH	AMeP	AMeP
11.0	CAPS – NaOH	Na_2CO_3 – $NaHCO_3$	CAPS	

The buffer which led to the higher activity and was used for data in Figure 2 is indicated here, unless activity was below the detection limit (< 0.02 μmol *p*NPP hydrolysed mg dry wt^{-1} h^{-1}). DMG, 3,3-dimethylglutaric acid; HEPES, N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; AMeP, 2-amino-2-methyl-1-propanol; and CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid.

The presence of EDTA at concentrations of 1 mM and above in the assay medium led to the complete inhibition of phosphomonoesterase activity (Table 2). However, when filaments which had been suspended in EDTA solutions of the same molarity were then tested in the normal assay medium, there was only a slight decrease in phosphomonoesterase activity (Table 2).

A higher concentration of NaOH was required to terminate cellular than extracellular activity; 0.3 M NaOH terminated extracellular activity, but reduced cellular activity by only 85%, and 1.5 M NaOH was required to terminate the latter effectively. SDS (1%) inhibited extracellular activity completely, but reduced cellular activity by only 20%.

Use of the Lineweaver-Burk plot, 1/v *versus* 1/s allowed the calculation of half-saturation values and concentration of ρNPP (K_m) required to support half the maximum rates. The K_m values for cellular and extracellular phosphomonoesterase were 4.34×10^{-5} M and 3.28×10^{-5} M, respectively.

Evidence that hairs are an important site of enzyme activity was obtained using a cellular fraction consisting of detached hairs (see Materials and methods). This showed very high activity, but the mass of hairs available was too low to obtain a rate. Several tests were carried out to establish whether cellular enzyme was

attached to a surface (such as cell wall or plasma membrane) or present in the periplasmic space. No significant increase in extracellular activity occurred when cellular material was exposed to trichloroethane (0.1 mM to 100 mM), 20% sucrose or lysozyme treatment. These all suggest that the enzyme is bound to a surface.

It seemed possible that enzyme activity in the extracellular fraction might be bound to colloidal carbohydrate material related to sheath carbohydrates. Ultracentrifugation (110,000 x *g*) of culture medium for 1 h removed 80% of the carbohydrate from the supernatant, but only 45% of enzyme activity, suggesting that at least part of the extracellular enzyme is truly soluble. However, staining of material for 15 min in a shaken snap-cap vial with BCIP showed localization of blue colour on mucilage, sheath and surface of the hair; the blue colour was

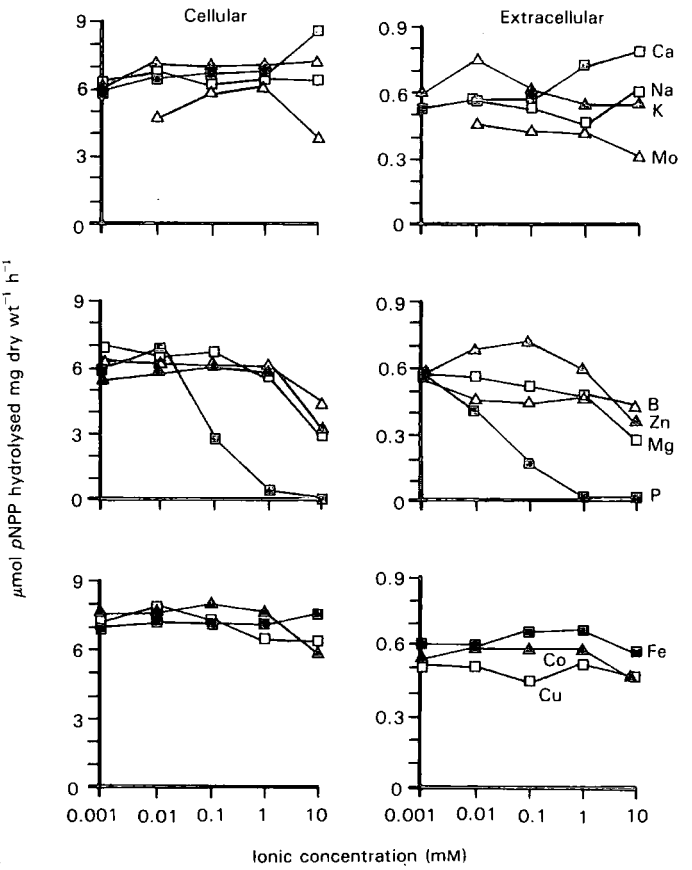

Figure 3 Influence of eleven ions on cellular and extracellular phosphomonoesterase activity in *Calothrix parietina* D550 shown by hydrolysis of *p*NPP. In order to use a logarithmic axis, the results for absence of ion are plotted as 0.001 M.

Table 2 Effect of EDTA on cellular phosphomonoesterase activity of *C. parietina* D550 during and prior to assay

Treatment	EDTA concentration (mM): 0.09		1		10		20	
	μmol *p*NPP hydrolysed mg dry wt^{-1} h^{-1}: $\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
During assay	5.82	0.27	<0.02		<0.02		<0.02	
Prior to assay	6.02	0.08	5.75	0.64	5.18	1.07	4.64	0.34

The slight decrease in activity associated with the pH drop at 20 mM EDTA is negligible. n = 8.

apparently due to a precipitate. Neither the surface of vegetative cells nor the cytoplasm were stained. When addition of BCIP was followed immediately by the material being placed on a slide under a coverslip, the staining reaction was quite different. The first coloration was seen within the hair by 5 min; by 15 min there was some blue colour on mucilage, sheath and hair surface, but less than when the cells were shaken in a vial. The intracellular coloration appeared initially to show a gradient from top to bottom of the filament, although it was difficult to be sure due to the photosynthetic pigments in the 'vegetative' cells.

Discussion

Phosphomonoesterase in *Calothrix parietina* is a stable enzyme, with cellular activity still detectable at 80°C, though not quite so stable as *E. coli*, where activity is detectable at 90°C (Torriani, 1960). Extracellular enzyme was less stable than cellular enzyme at high temperatures and also slightly more sensitive to many other environmental factors. Somewhat in contrast, 'cell-free' enzyme of *Plectonema boryanum* (Doonan and Jensen, 1980), obtained by lysis of cellular material with polymixin B, had a greater thermal stability than cellular (whole filament) activity. The pH optimum of phosphomonoesterase activity in *C. parietina* (pH 10—10.2) is towards the upper end of the range observed for other cyanobacteria (pH 8—10: Healey, 1973; Ihlenfeldt and Gibson, 1975; Healey and Hendzel, 1979), although another strain of *Calothrix* (*C. viguieri*) isolated from the upper part of the surface of a mangrove root has a pH optimum for cellular phosphomonoesterase activity of 12.2 (unpublished data).

The ionic requirements reported for microbial phosphomonoesterases vary considerably. In no case did the absence of a particular ion in assay medium lead to a marked reduction in activity with *C. parietina*. Calcium had the greatest stimulatory effect, but activity of thoroughly washed cellular and extracellular material remained high even in its absence. Calcium has been reported to be a requirement for alkaline phosphomonoesterase activity in many micro-organisms,

e.g. Micrococcus sodonensis (Glew and Heath, 1971) and *Anabaena variabilis* (Healey, 1973). Magnesium had a slight inhibitory activity at all concentrations on extracellular activity of *C. parietina*, although it has been reported to be stimulatory or have no effect in other micro-organisms. Phosphomonoesterases of *Escherichia coli* (Schlesinger *et al.*, 1969), *Aspergillus niger* (Dorn, 1968) and *Pseudomonas aeruginosa* (Day and Ingram, 1973) all show enhanced activity in the presence of magnesium. The inhibitory effect of EDTA is similar to that found in other studies on phosphomonoesterase (*e.g.* Whitt and Savage, 1988).

Inorganic phosphate (P_i) inhibited phosphomonoesterase activity in *C. parietina*, which is a feature common to all inducible phosphomonoesterase systems (Torriani, 1960; Healey, 1973; Ingram *et al.*, 1973; Ihlenfeldt and Gibson, 1975). At 1 mM P_i, 5% of activity remained and at 10 mM there was no activity. *C. parietina* is more sensitive to P_i than *Plectonema boryanum* (Doonan and Jensen, 1980) and *E. coli* (Torriani, 1960) where 30% of activity remained at 10 mM P_i. However, inhibition of activity during routine assays with *C. parietina* is negligible, because, even if all P_i released remained in the medium, the maximum concentration reached at the end of 20 min assays would be about 2 μM P_i. The K_m values for ϱNPP of 4.34×10^{-5} M and 3.28×10^{-5} M with cellular and extracellular phosphomonoesterase are similar to values for many other micro-organisms, *e.g. E. coli* (1.2×10^{-5} M; Garen and Levinthal, 1960), but an order of magnitude less than *Anabaena variabilis* (7×10^{-4} M; Healey, 1973). The concentrations at which the environmental variables had a detectable effect during assays may be compared with concentrations found in culture media and in the stream from which the organism was isolated. The most obvious differences are for temperature and pH. Temperature optima in laboratory assays (Figure 1) were above temperatures ever likely to be encountered in the stream ($x = 8.5°C \pm 6.2$) and for most of the year activity may be expected to be less than 30% of the maximum. The organism developed phosphomonoesterase during growth in medium at a pH (7.0) at which activity is not expressed.

The stream, which has been sampled at monthly intervals over 1 year (Holmes and Whitton, 1981), showed a 'mean' pH of 7.8 (SD $\pm$ 0.4), a pH value at which laboratory activity is about 40% of the maximum (pH 10.2). However, it is difficult to relate behaviour in the field to that in the laboratory, because water in this stream shows the highest organic phosphate concentration at a season when the contribution from peat drainage is highest and pH values are lowest. The inhibitory effect of EDTA suggests the importance of one or more metals in the enzyme structure, but the only element present in the environment likely to influence activity directly is Ca. The concentration of Ca in stream water is 1 $\pm$ 0.47 mM, sufficient to raise extracellular, but not cellular, activities.

The differing responses to BCIP in shaken vials and under a coverslip may perhaps be due to differing availability of oxygen. Staining by BCIP results from two separate reactions (Coston and Holt, 1958). BCIP is first hydrolysed, releasing one molecule of soluble, colourless indole and one molecule of phosphate; this is followed by an oxidation step, which leads to the insoluble blue indigoid pigment, 5-bromo-4-chloro-indigo. Staining is therefore dependent both on

enzyme activity and the presence of oxygen. The initial O_2 concentration is about 0.15 mM (at 32°C), whereas BCIP is supplied at 1 mM. Released indole will scavenge O_2. Unless photosynthetic oxygen evolution compensates for this, it will lead to a reduced concentration under the coverslip, whereas the saturation concentration will be maintained in the shaken vial. It is suggested that under the coverslip indole enters the cell and is oxidized in cytoplasm by O_2 resulting from photosynthesis.

This is the first study to compare cellular and extracellular phosphomonoesterase activity in a cyanobacterium. In general the enzymes have very similar properties, although extracellular activity is slightly more sensitive to environmental factors. Purified enzymes from the two fractions will be required to demonstrate whether or not enzyme from both sources has an identical structure.

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